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Investigating the role of Akt1 in prostate cancer development through phosphorylation-dependent regulation of Skp2 stability and oncogenic function

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14. ABSTRACT

Elevated Skp2 expression is frequently observed in many tumors including breast and prostate carcinomas. However, the molecular mechanisms underlying elevated Skp2 expression in prostate cancers have not been fully explored. Hyperactivation of the Akt pathway is considered a hallmark of many cancers and it has been reported that activation of the PI3K/Akt pathway enhances p27 destruction. Thus, we hypothesize that sustained Akt activity in prostate cancer cells leads to elevated phosphorylation of Skp2, and subsequently influences Skp2 stability and its oncogenic functions. In support of our hypothesis, we found that Skp2 abundance is affected by manipulation of the PTEN/PI3K/Akt pathway in the PC3 and LNCaP prostate cancer cell lines. Furthermore, we showed that this is partially through phosphorylation of Skp2 by Akt, which impairs Skp2 destruction by Cdh1. Sequence analysis revealed that the Akt phosphorylation site (Ser72) is localized in a putative Nuclear Localization Sequence (NLS). Consistent with this notion, we found that overexpression of Myr-Akt promoted Skp2 cytoplasmic translocation, and that inhibition of PI3K/Akt activity enhanced its nuclear localization. We believe that these studies will provide a novel mechanism for Skp2 overexpression in prostate cancers and provide the rationale for developing Akt1-specific inhibitors to treat prostate cancer patients.

15. SUBJECT TERMS

Akt1, PI3K, phosphorylation, Skp2, ubiquitination, oncogene, Cdh1, NLS, cytoplasmic

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Introduction

Project Title: Investigating the role of Akt1 in prostate cancer development through phosphorylation-dependent regulation of Skp2 stability and oncogenic function

Defective cell cycle regulation leads to genomic instability, which ultimately contributes to cancer development (3, 9). Two related, multi-subunit E3 ubiquitin ligase enzymes, the Anaphase Promoting Complex (APC) and the Skp1-Cullin1-F-box (SCF) complex are the major driving forces that govern ordered cell cycle progression through promoting the destruction of the key cell cycle regulators (8). Elevated Skp2 expression is frequently observed in many tumors including breast and prostate carcinomas (5, 11). It has been proposed that enhanced Skp2 expression leads to the accelerated degradation of targets such as p27 (4, 12) and other cell cycle regulators including FOXO1 and Orc1, thus promoting cell cycle progression and favoring transformation (3). Furthermore, ectopic overexpression of Skp2 facilitates transformation of Rat1 cells in soft agar and in nude mouse xenograft assays (5). The oncogenic potential of Skp2 is further illustrated by developing prostate and lymphoma cancers in Skp2 transgenic mice (6, 10). However, the molecular mechanisms underlying elevated Skp2 expression in prostate cancers have not been fully explored. We and others have identified Cdh1 as the E3 ligase that promotes Skp2 destruction (2, 14). In contrast to the frequency of Skp2 overexpression, loss of Cdh1 is not a frequent event; thus it cannot explain the observation of elevated Skp2 levels in carcinomas. On the other hand, hyperactivation of the Akt pathway is considered a hallmark of many cancers including prostate cancer and it has been reported that activation of the PI3K/Akt pathway enhances p27 destruction (13). This suggests that sustained Akt activity can influence Skp2 activity. Consistent with this, studies have also demonstrated that Akt can contribute to Skp2 overexpression, although the mechanism has not been explored (1, 7). We noticed that the Skp2 protein contains a canonical Akt phosphorylation site. Thus, we hypothesize that sustained Akt activity in prostate cancer cells leads to elevated phosphorylation of Skp2, and subsequently influences Skp2 stability and its oncogenic functions. The proposal aims to determine how Akt1 phosphorylation of Skp2 disrupts its destruction mediated by the APC/Cdh1 complex and to dissect the molecular mechanisms by which Akt1 promotes Skp2 cytoplasmic localization in prostate cancer cells. Furthermore, we would like to evaluate whether specific inhibition of Akt1 can be used as a novel treatment for prostate cancer patients.

Body

Specific Aim 1: Determine how Akt1 phosphorylation of Skp2 disrupts its destruction mediated by the APC/Cdh1 complex.

Task 1: To determine whether disregulated PTEN/PI3K/Akt pathway contributes to the elevated Skp2 expression in prostate cancer (Month 1-6).

a. *To examine whether Skp2 abundance positively correlates with Akt activity, as determined by the p-Ser473 Akt signal, in a panel of prostate cancer cells including DU145 cells with normal expression of the PTEN protein, as well as LNCaP, PC3 and PC346 cells that lack the expression of PTEN (Month 1-3).*

We found that comparing with DU145 cell line with wild-type PTEN, PC3 cells which lack PTEN expression, have elevated Skp2 expression. However, we have not extended this finding to a big panel of prostate cancer cell lines to examine whether this is a generalized phenomenon. We are in the process of collecting the panel of prostate cancer cell lines to fully investigate it.

b. To further examine whether inhibition of PI3K/Akt activity leads to downregulation of Skp2 in PTEN-null cell lines (Month 2-3).

As illustrated in Fig. 1, we found that inactivating PI3K/Akt pathway in PC3 cells leads to a severe reduction of Skp2 expression that correlates well with reduction in Akt activity.

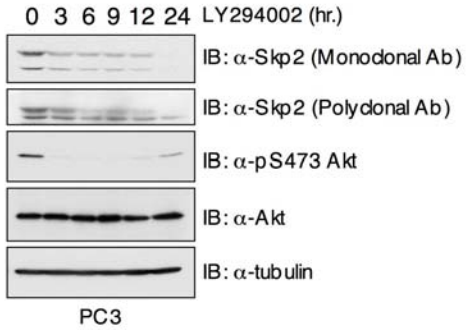

Figure 1: Inactivation of the PI3K/Akt pathway leads to downregulation of Skp2 expression levels in PC3 prostate cancer cell lines with elevated Akt activity.

Immunoblot (IB) analysis of whole-cell extracts (WCE) derived from PC3 cells treated with PI3K inhibitor LY294002 for indicated time.

c. Milestone: To examine whether knock-down of Akt1 or Akt2 with shRNA lentiviral constructs in prostate cancer cells affects Skp2 expression (Month 3-6).

We found that in both HeLa (data not shown) and SKBR3 cell lines (Fig. 2), depletion of endogenous Akt1, but not Akt2, leads to a significant decrease of Skp2 protein abundance. These results support the hypothesis that PI3K/Akt pathway governs Skp2 stability. We have not infected PC3 or LNCaP prostate cell lines to examine whether similar mechanism also exists in the prostate cancer settings. However, results from Fig. 1 suggested that PI3K/Akt activity is also involved in regulating Skp2 levels in prostate cancer cells.

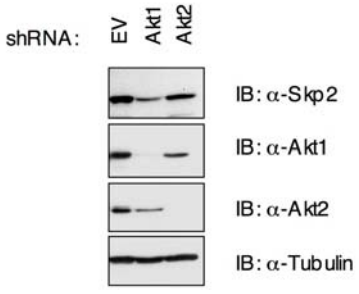

Figure 2: Depletion of Akt1, but not Akt2, results in a sharp decrease in Skp2 expression levels in SKBR3 cells.

SKBR3 cells were infected with indicated lenti-viral shRNA vectors, and then incubated with 2 μg/ml puromycin for at least four days to eliminate the non-infected cells. Cell lysates were collected and immunoblots were performed with indicated antibodies.

Task 2: To determine whether Ser72 is the major physiological Akt1 phosphorylation site (Month 6-12).

a. Immunoprecipitate endogenous Skp2 protein from PC3 cell line and perform mass spectroscopy analysis on the recovered Skp2 immunoprecipitate to analyze its phosphorylation status (month 7-8).

We purchased Skp2 antibody from both Santa Cruz (rabbit anti-Skp2 polyclonal antibody) and also from Zymed (mouse anti-Skp2 monoclonal antibody). We performed endogenous Skp2 IP and then run the immunoprecipitate on SDS-gel and stained with gel-code blue reagent (from Pierce). We found that both Skp2 antibodies are not very efficient in immunoprecipitating endogenous Skp2. Since there are no better Skp2 antibodies available, we think that it might be a better approach to ectopically express HA-Skp2 and then perform Mass Spectrometry on the recovered HA-Immunoprecipitate.

b. Alternatively, we will generate a 293T cell line stably expressing HA-Skp2 using retroviral infection, and perform HA-IP and perform mass spectrometry analysis on the recovered HA-immunoprecipitate (month 7-9).

We transiently transfect 293T cells with HA-Skp2 mammalian expression plasmid, and after 48 hours, the transfected 293T cells were harvested for HA-IP. After extensive washing conditions, HA-immunoprecipitates were separated on SDS-PAGE gel and then stained with gel-code blue reagent. The band containing Skp2 was excised and sent for mass spectrometry analysis.

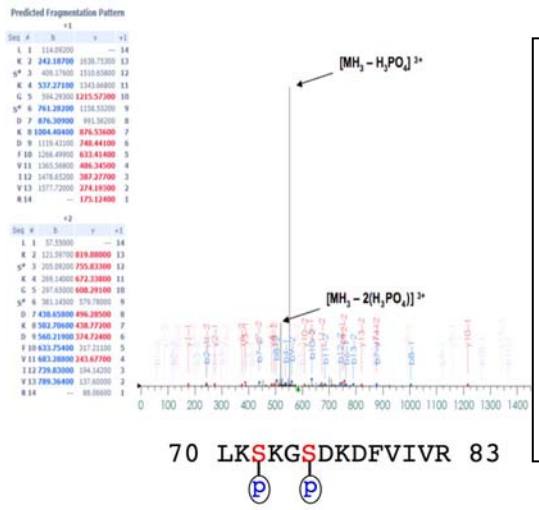

Figure 3. Detection of *in vivo* Skp2 phosphorylation status by mass spectrum analysis.

HA.Skp2 was transfected into 293T cells, then immunoprecipitated with anti-HA in the presence of phosphatase inhibitors. The immunoprecipitate was resolved by SDS-PAGE and phosphorylation was detected by mass spectrum analysis. The symbol # indicates the site of phosphorylation. Ser72 and Ser75 sites were detected to be phosphorylated *in vivo*.

c. To determine whether depletion of endogenous Akt1 and Akt2 affects Ser72 phosphorylation of Skp2 (month 9-11).

As shown in Figure 4, in collaboration with Cell Signaling company, we are in the process of developing the p-Ser72-Skp2 antibody. Now we are examining whether this antibody could detect the endogenous level of Skp2 Ser72 phosphorylation status. This will be an important tool for us to further investigate whether Skp2 phosphorylation at Ser72 will be affected by depletion of Akt1 or Akt2.

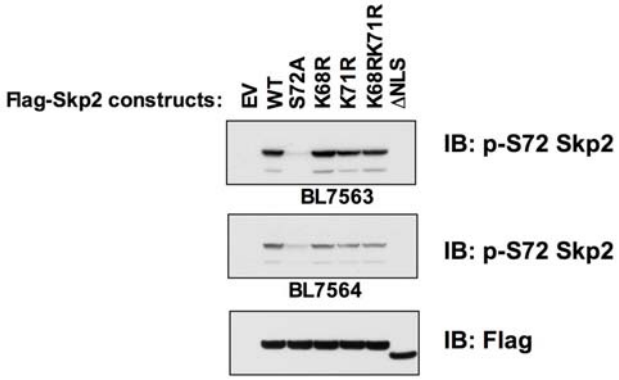

Figure 4: Development of antibody that can detect Skp2 Ser-72 phoshorylation status.

Various Flag.Skp2 constructs were transfected into 293T cells, 48 hours later, cell lysis were collecated in the presence of phosphatase inhibitors. Immunoblots were performed with the indicated antibodies.

d. Using in vitro kinase assay and mass spectrometry analysis to reveal the exact site where Skp2 protein is phosphorylated after the addition of Akt1 in vitro (month 8-10).
We performed *in vitro* kinase assay with active Akt1 and recombinant GST-Skp2 protein, and then send the products for mass spectrometry analysis. However, no phosphorylation was detected, possibly due to the fact that the phosphorylation efficiency is too low and only a very small percentage of GST-Skp2 was phosphorylated. In order to get this assay to work, we have to enhance the *in vitro* kinase assay efficiency.

e. *Milestone: We plan to generate GST-Skp2 fusion proteins whose individual potential Akt phosphorylation sites have been point-mutated which will allow us to use in vitro kinase assay to determine whether Ser72 is the major phosphorylation site (month 10-12).*
Using the Scansite program, we performed bioinformatic scan of Skp2 protein primary sequence and found that besides the high stringency Akt site at Ser72, Skp2 contact two other low stringency Akt sites at Ser75 and Thr21. We generated GST-Skp2 mutants as listed in Figure 5 and performed *in vitro* kinase assay. We found that only after mutation of Ser72 site, the Akt-mediated Skp2 phoshorylation is severely reduced, indicating that Ser72 is the only major Akt phosphorylation site present in Skp2.

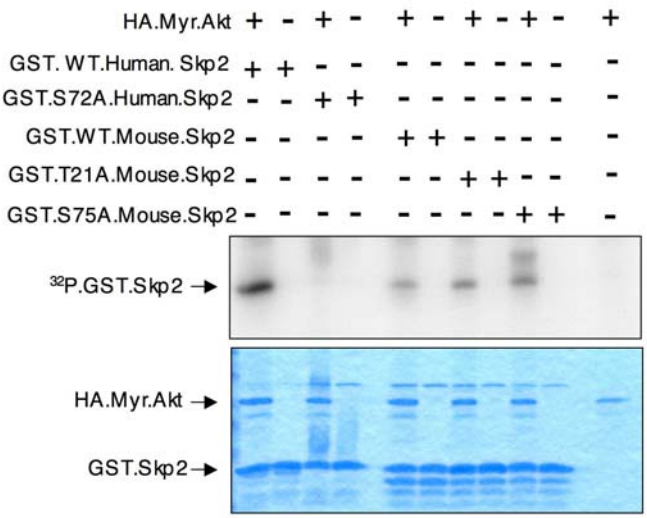

Figure 5. *In vitro* phosphorylation of the mouse Skp2 protein by Akt.

HA.Myr.Akt was transfected into 293T cells, then immunoprecipitated with anti-HA and incubated with 5 μg of indicated GST.Skp2 in the presence of γ-^{32}P-ATP. The kinase reaction products were resolved by SDS-PAGE and phosphorylation was detected by autoradiography.

Task 3: To determine how Akt1 phosphorylation of Skp2 at Ser72 impairs its destruction by APC/Cdh1 (Month 12-18).

a. We will use real-time RT-PCR to compare the levels of Skp2 mRNA before and after specific Akt1 depletion in the PC3 prostate cancer cells and HeLa cells (Month 12-14).
We found that in HeLa cells, inactivation of Akt1, but not Akt2 will dramatically reduce Skp2 protein abundance, and only moderately reduce Skp2 mRNA levels. These results indicate that Akt1 could influence both Skp2 protein stability and Skp2 transcription, however, Akt1 might mainly govern Skp2 activity in a post-translational mechanism. We will perform similar experiments in PC3 cells to investigate whether Akt1-mediated Skp2 regulation mainly involves a post-translational mechanism.

b. We will measure the changes in Skp2 protein half-life after depletion of Akt1 in PC3 prostate cancer cells and HeLa cells, using GFP shRNA treatment as a negative control (Month 12-14).
As illustrated in Figure 6, we found that in HeLa cells, Skp2 protein half-life is tightly controlled by the PI3K/PTEN/Akt pathway. We will perform similar experiments in PC3 cells to investigate whether the Skp2 stability in prostate cancer cells are also governed by the PI3K/Akt pathway. However, since our preliminary data suggested that the siRNA transfection efficiency in PC3 cells are much lower than HeLa cells. As an alternative approach, we will infect PC3 cells with shRNA lenti-viral vector and then use puromycin treatment to eliminate the non-transfected cells before the CHX half-life experiments.

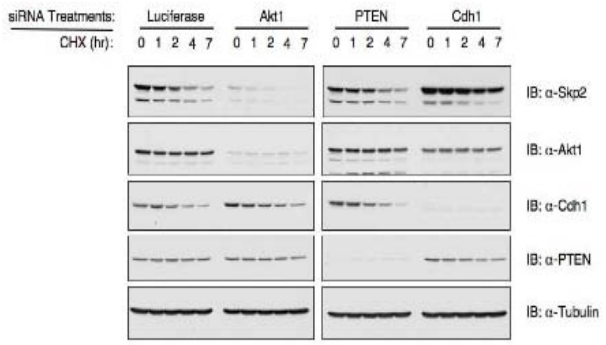

Figure 6: PI3K/Akt pathway regulates Skp2 stability in HeLa cells.
HeLa cells were transfected with the indicated siRNA oligos. After 40 hours, cells were treated with 20 μg/ml CHX. At the indicated time points, whole-cell lysates were prepared and immunoblots were performed with indicated antibodies.

c. Milestone: We will perform Akt1, Cdh1 and Akt1/Cdh1 double RNAi treatment (with mock and luciferase RNAi as negative controls) in synchronized HeLa cells, and then measure any changes in Skp2 protein abundance and cell cycle progression. However, both Akt1 and Cdh1 play important roles in cell cycle regulation and Skp2 expression levels fluctuate considerably throughout the cell cycle (Month 12-16).
We found that depletion of Akt1 leads to Skp2 downregulation while depletion of Cdh1 leads to Skp2 upregulation. More importantly, depletion of both Akt1 and Cdh1 leads to restoration of Skp2 abundance, indicating the Akt1 regulates Skp2 stability in a Cdh1-dependent manner. We plan to perform similar experiments in PC3 cells and as described previously, it might be difficult to achieve high-efficiency siRNA transfection. To overcome this pitfall, we will rely on shRNA lentiviral infection to deplete endogenous Akt and Cdh1.

d. Milestone: To determine whether the interaction between Cdh1 and Skp2 will be disrupted by Akt phosphorylation at Ser72 (Month 15-17).

As demonstrated in Figure 7, we found that in the *in vitro* GST-pull down experimental setting, mutating Ser72 and Ser75 to Asp to mimick phosphorylation event significantly reduce Cdh1 and Skp2 interaction. We plan to also perform *in vivo* co-immunoprecipitation assays to provide more supporting evidence for this finding.

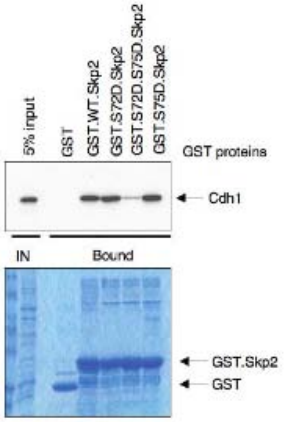

Figure 7: Phosphorylation of Skp2 by Akt1 impairs Skp2 interaction with its E3 ubiquitin ligase Cdh1. Autoradiography of ^{35}S-labelled Cdh1 bound to the indicated GST-Skp2 protein.

e. We will utilize an in vivo ubiquitination assay to demonstrate that the ability of APC/Cdh1 to promote Skp2 ubiquitination is compromised by Akt (Month 16-18).

We are currently optimizing the experimental conditions to show that Skp2 could be efficiently ubiquitinated *in vivo* by APC/Cdh1 and furthermore, this process is inhibited by the PI3K/Akt signaling pathway.

Key Research Accomplishments:

- We found that Skp2 is phosphorylated by Akt1 at Ser72
- We also showed that inactivation of PI3K/Akt pathway in PC3 prostate cancer cells leads to downregualtion of Skp2 protein abundance
- We further showed that Akt1-mediated phosphorylation of Skp2 leads to stabilization of Skp2, this finding offers a novel explanation for the frequent Skp2 overexpression in prostate cancers.
- We further illustrated that phosphorylation of Skp2 by Akt impairs Skp2/Cdh1 interaction, thus allowing Skp2 to escape Cdh1-mediated destruction.
- We also generated an antibody that can recognize the p-Ser72 form of Skp2, which will be very useful reagent to further explore how Akt1 regulate Skp2 phosphorylation to affect Skp2 oncogenic activity.

Reportable Outcomes:

We published two relevant papers as listed below:

1. Gao, D., Inuzuka, H., Tseng, A., **Wei, W.** (2009) Akt finds its new path to regulate cell cycle through modulating Skp2 activity and its destruction by APC/Cdh1. ***Cell Division*** 4:11.
2. Gao, D., Wan, L., Inuzuka, H., Berg, A. H., Tseng, A., Zhai, B., Shaik, S., Bennet, E., Tron, A. E., Gasser, J.A., Lau, A., Gygi, S., Harper, J. W., DeCaprio, J. A., Toker, A. and **Wei, W.** (2010) Rictor forms a complex with Cullin-1 to promote SGK1 ubiquitination and destruction. ***Molecular Cell*** 39(5): 797-808

Conclusions: We showed that Akt1, but not Akt2, directly controls Skp2 stability by a mechanism that involves degradation by the APC/Cdh1 ubiquitin ligase complex. Furthermore, we showed that in prostate cancer cell line PC3, inactivation of PI3K/Akt leads to downregulation of Skp2 protein levels. This is partially due to the fact that phosphorylation of Skp2 by Akt impairs Skp2 interaction with its E3 ligase Cdh1, allowing Skp2 to escape Cdh1-mediated proteolysis. In addition, we found that Ser72 is localized within a putative Nuclear Localization Sequence (NLS) and thus we also propose that phosphorylation of Ser72 by Akt might lead to Skp2 cytoplasmic translocation.

We believe that our proposed studies will provide a novel mechanism to explain the observations of Skp2 overexpression in prostate cancers. They will also expand our knowledge of how specific kinase signaling cascades influence proteolysis governed by APC/Cdh1 complexes, and will provide further evidence that elevated Akt activity is responsible for the cytoplasmic Skp2 staining which is observed in more aggressive and advanced cases of prostate cancer. These studies should allow us to assess whether inhibition of the PI3K/Akt pathway could be a novel and therapeutically promising way to block the growth of prostate tumor cells by downregulation of Skp2. Furthermore, our work will provide the rationale for developing Akt1-specific inhibitors as efficient anti-cancer drugs for prostate cancer patients.

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Appendices:
Enclosed please find the reprint of the two published articles.

Supporting Data:
None

Cell Division

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Review

Open Access

Akt finds its new path to regulate cell cycle through modulating Skp2 activity and its destruction by APC/Cdh1

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Abstract

Skp2 over-expression has been observed in many human cancers. However, the mechanisms underlying elevated Skp2 expression have remained elusive. We recently reported that Akt1, but not Akt2, directly controls Skp2 stability by interfering with its association with APC/Cdh1. As a result, Skp2 degradation is protected in cancer cells with elevated Akt activity. This finding expands our knowledge of how specific kinase cascades influence proteolysis governed by APC/Cdh1 complexes. However, it awaits further investigation to elucidate whether the PI3K/Akt circuit affects other APC/Cdh1 substrates. Our results further strengthen the argument that different Akt isoforms might have distinct, even opposing functions in the regulation of cell growth or migration. In addition, we noticed that Ser72 is localized in a putative Nuclear Localization Sequence (NLS), and that phosphorylation of Ser72 disrupts the NLS and thus promotes Skp2 cytoplasmic translocation. This finding links elevated Akt activity with the observed cytoplasmic Skp2 staining in aggressive breast and prostate cancer patients. Furthermore, it provides the rationale for the development of specific Akt1 inhibitors as efficient anti-cancer therapeutic agents.

Introduction

In dividing cells, the cell cycle is tightly controlled by multiple regulatory mechanisms to ensure that DNA is faithfully replicated only once in the S phase and then distributed equally between two daughter cells in the M phase. Defective cell cycle regulation can lead to genomic instability, which ultimately facilitates cancer development. Many key regulators governing the cell cycle progression are short-lived proteins, and selective degradation of these regulators by the ubiquitin-proteasome system has recently been shown to be a major mechanism for ensuring ordered and coordinated cell cycle progression [1,2]. Moreover, the irreversible nature of proteolysis guarantees the uni-directional execution of the cell cycle program, driving the cell cycle from one stage to the next. There are two related, multi-subunit E3 ubiquitin ligase enzymes, the Anaphase Promoting Complex (APC) and the Skp1-Cullin1-F-box complex (SCF) that are considered to be the major driving forces governing proper cell cycle progression [3]. SCF is active from the late G1 phase until the G2 phase and mediates the ubiquitination of G1 cyclins and Cdk inhibitors. SCF consists of the invariable components Skp1, Cul1, and Rbx1, as well as a variable component known as an F-box protein that is responsible for substrate recognition. There are 68 putative F-box proteins encoded in the human genome which can form individual SCF complexes, each with different F-box proteins incorporated into the core Skp1/Cullin-1-Rbx1 complex [4]. The diversity of these SCF complexes ultimately provides the high stringency neces-

sary for substrate specificity. The well-characterized F-box proteins Skp2, Cdc4/Fbw7, and β-Trcp1 target p27 [5], cyclin E [6,7], and Cdc25A [8,9], respectively, for ubiquitination and degradation. In all cases, proper phosphorylation of the substrate is required for interaction with the F-box proteins. Unlike SCF, APC is active from the late G2 phase to the mid-G1 phase, and is responsible for the degradation of mitotic cyclins, securin, and geminin. Although APC is composed of 11 subunits, the general structure is very similar to SCF. The substrate adaptors Cdc20 and Cdh1 are equivalent to the F-box proteins, but both Cdh1 and Cdc20 do not require post-translational modification of their respective substrates for recognition, instead, binding to their substrates via Destruction Boxes (D-Box) or KEN Boxes.

Skp2 was originally identified as an S-phase Kinase Cdk2/Cyclin A-associated protein [10]. Subsequently, the identification of an F-box domain within its coding sequence suggested the presence of E3 ubiquitin ligase activity [5,11]. Besides its major downstream target p27, recent studies have demonstrated that the Skp2/SCF complex also targets numerous other substrates for degradation, many of which are negative cell cycle regulators. These include p21, p57, p130 and FOXO1 [1]. p27 functions as a tumor suppressor such that its inactivation predisposes mice to cancer development [12]. However, in contrast with known tumor suppressor genes such as p53 or Rb, homozygous loss or silencing of the p27 gene is rarely found in human cancers. Instead, it is reduced p27 protein expression which is often linked to human malignancy, suggesting that regulation occurs mainly at the post-translational level [13]. Indeed, elevated Skp2 expression is frequently observed in many tumors including breast and prostate carcinomas [14,15]. It has been proposed that enhanced Skp2 expression leads to the accelerated degradation of targets such as p27 and other cell cycle regulators, thus promoting cell cycle progression and favoring transformation. Furthermore, overexpression of Skp2 facilitates transformation of Rat1 cells in soft agar and in nude mouse xenografts [14]. The oncogenic potential of Skp2 is further illustrated in transgenic mice. In one report, overexpression of Skp2 in the mouse prostate induced hyperplasia, dysplasia and low-grade carcinoma [16], while others have reported that Skp2 transgenic mice co-expressing N-Ras develop lymphomas [17]. These findings support the contention that Skp2 overexpression inversely correlates with low p27 expression, and positively correlates with tumor malignancy and poor diagnosis.

However, the molecular mechanisms underlying elevated Skp2 expression have not been fully explored. We and others have previously demonstrated that Cdh1 is the upstream E3 ubiquitin ligase which promotes Skp2 destruction [18,19]. In contrast to the frequency of Skp2 overexpression, loss of Cdh1 is not a frequent event in human cancer. Thus, loss of Cdh1 cannot explain the observation of elevated Skp2 levels in carcinomas. On the other hand, hyperactivation of the Akt pathway through various means of genetic alterations is considered a hallmark of many cancers. Furthermore, it has been reported that activation of the PI 3-K (phosphoinositide 3-kinase)/Akt pathway enhances p27 destruction [20]. This suggests that sustained Akt activity can influence Skp2 activity. Consistent with this, studies have also demonstrated that Akt can contribute to Skp2 overexpression, although the mechanism has not been explored [21,22].

The Akt family of kinases is composed of three closely related family members designated Akt1, Akt2 and Akt3, also known as PKBα (Protein Kinase B), PKBβ and PKBγ, respectively. Akt isoforms are known to play critical roles in many cellular processes including proliferation, transformation, survival and metabolism [23]. The PI 3-K and Akt pathway is frequently amplified and hyperactivated in most human cancers [24]. Upon activation of receptors for growth factors such as IGF-1 (insulin-like growth factor-1), activation of PI 3-K leads to the synthesis of the second messenger PtdIns-3,4,5-P_3 which binds and recruits Akt to the plasma membrane. Phosphorylation of Akt by upstream kinases fully activates the enzyme allowing it to phosphorylate multiple substrates which contain a minimal motif usually comprising RxRxxS/T (hereby x is any amino acid) [23]. Akt activity is negatively regulated by two tumor suppressors, the PTEN lipid phosphatase which dephosphorylates PtdIns-3,4,5-P_3 [25], and PHLPP, a Ser/Thr phosphatase which dephosphorylates Akt at Ser473, leading to its inactivation [26]. Since most of the upstream regulators and downstream mediators of the Akt pathway are either oncogenes or tumor suppressors, it is not surprising to find that Akt activity is abnormally elevated in most human cancers [27]. Major mechanisms described to date which explain hyperactivation of Akt include loss-of-function mutations in PTEN, as well as gain-of-function mutations in upstream regulators such as the receptors HER2, EGF-R and Ras [28]. In addition, constitutively active PI 3-K mutations have been identified in several human cancers and shown to be causally linked to elevated Akt signaling [29]. Enhanced Akt signaling in tumor cells can suppress apoptosis by promoting the phosphorylation and subsequent cytoplasmic localization of many downstream pro-apoptotic target proteins such as Bad [30], FOXO1 [31] and FOXO3a [32]. Akt upregulation can also promote cell growth by inactivating the negative cell cycle regulators p21 [33] and p27 [34-36]. Most studies that have explored a role for the PI 3-K and Akt pathway in cell cycle progression, survival and cancer progression have generally assumed that all three isoforms function in a overlapping and redundant

manner. However, recent studies have begun to suggest isoform-specific functions for Akt. This was first highlighted by distinct phenotypes born out from the Akt1 and Akt2 knockout mice [37]. At the level of signaling and cell cycle progression, Akt1 has been shown to promote cell cycle progression, whereas Akt2 promotes cell cycle exit in myoblasts [38]. The PHLPP1 and PHLPP2 isoforms differentially dephosphorylate Akt1 and Akt2 leading to distinct accessibility of each Akt isoform to substrates such as p27, FOXO3a and GSK-3 [39,40]. Finally, Akt1 and Akt2 have been shown to function in an opposing manner in the regulation of breast cancer cell invasive migration. (Irie et al., 2005).

The Skp2 Ser72 Akt phosphorylation site is conserved in large mammals

Following these clues, we and others recently demonstrated that elevated Akt activity could positively influence Skp2 activity, and impair its destruction by the APC/Cdh1 E3 ubiquitin ligase complex [41,42] (Figure 1). At first glance, this offers a molecular mechanism for the frequently observed Skp2 overexpression in many human cancers and links this phenomenon to the abnormal activation of the PI 3-K and Akt pathway, which is well-documented as being aberrantly activated in the majority of human cancers. The Ser72 site is conserved in most large mammals we examined, including primates, dogs, horses, pigs, cows, and even rats. However, this putative Ser72 Akt phosphorylation site is not present in the mouse sequence. It is known that similar inter-species differences also exist for other Akt substrates including p27 [36] and caspase-9 [43]. We further found that the Ser72 site is not conserved in Xenopus or Zebrafish, thus indicating that the Akt/Skp2 regulatory pathway might have evolved as a gain-of-function event later in evolution. On the other hand, it is well-established that tumorigenesis differs dramatically between humans and mice [44]. Hence, it is plausible that for large animals with a longer life span than mice, which requires more cell division events, an additional layer of cell cycle control is developed. It is also

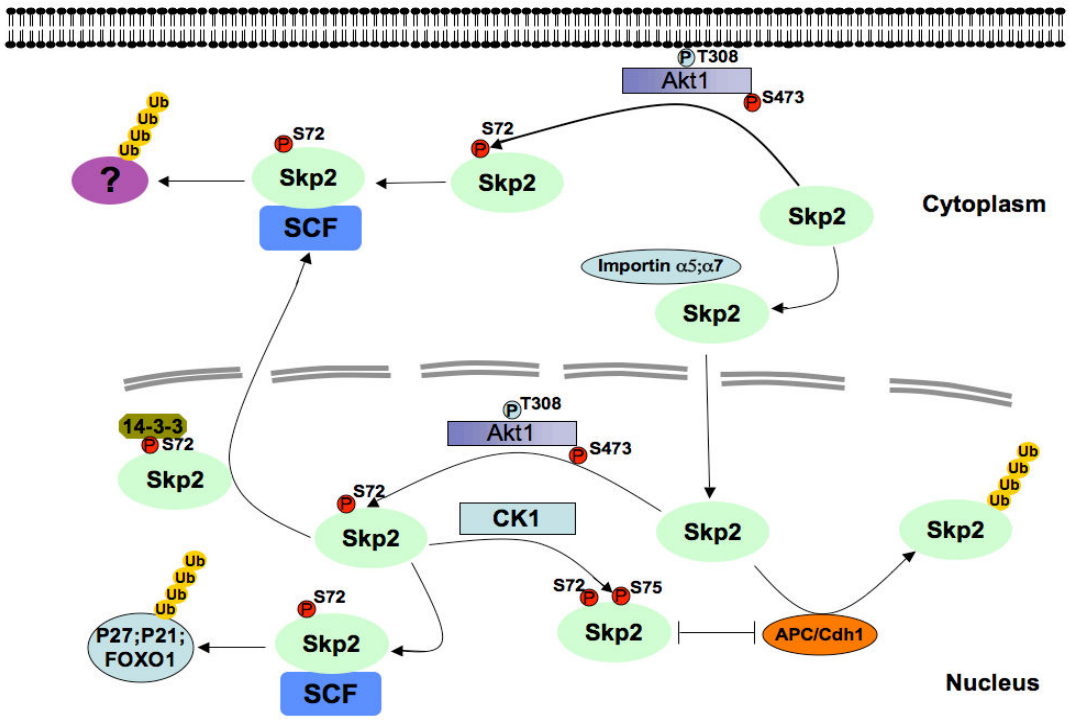

Figure 1

Schematic model for how Akt1-dependent phosphorylation of Skp2 at the Ser72 site promotes Skp2 cytoplasmic localization and stabilizes Skp2 by impairing its association with the APC/Cdh1 E3 ubiquitin ligase complex. Phosphorylation of Skp2 at Ser72 by Akt1 greatly reduces its ability to interact with the importin complex as well as promotes its association with 14-3-3, resulting in cytoplasmic retention. Furthermore, phosphorylation of Skp2 by Akt1 primes Skp2 for subsequent phosphorylation of Ser75 by Casein Kinase I (CKI). Phosphorylation on both Ser72 and Ser75 results in impaired association with Cdh1, thus allowing Skp2 to escape APC/Cdh1-mediated ubiquitination and destruction.

possible that there is another universal molecular mechanism shared by most species to control Skp2 stability/activity, which is not identified yet.

The potential role for Casein Kinase I in Skp2 stability control

Our results further demonstrate that phosphorylation of human Skp2 at Ser72 creates a priming site, and that CKI may be one of the kinases which phosphorylates Ser75, a process that would lead to subsequent dissociation from Cdh1 and stabilization of Skp2. Sequence analysis also reveals that the Ser75 site is not conserved in all mammals we examined; although most large mammals contain Ser72, they do not contain Ser75, which is replaced by an Asn. This indicates that CKI or other Ser75 kinases are not likely to efficiently phosphorylate Skp2 in these species. Clearly additional studies are required to examine in more detail the regulation of Ser75 phosphorylation in human Skp2, and to determine if CKI is the physiologically-relevant kinase, and similarly whether Akt regulates Skp2 stability in species that do not harbor Ser75.

Regardless, our data clearly points to an important distinction between human and mouse Skp2 regulation by the Akt pathway. First, phosphorylation of Ser72 is critical for the ability of Akt to regulate Skp2 stability. Since mouse Skp2 does not contain this site, it explains why mouse Skp2 expression is not affected by the Akt pathway. Secondly, additional experiments using human Skp2 have revealed distinct contributions of phosphorylation events to Skp2 stability. Phosphorylation of Ser72 by Akt is sufficient to disrupt the association between importin and Skp2, leading to Skp2 cytoplasmic translocation. Moreover, phosphorylation of both Ser72 and Ser75 by Akt1 and CKI, respectively, is required to disrupt the association between Cdh1 and Skp2, thus stabilizing Skp2. However, since Cdh1 is primarily localized in the nucleus, phosphorylation of Skp2 by Akt itself might be sufficient to stabilize a portion of Skp2 by cytoplasmic translocation.

Akt1 regulates both Skp2 stability and Skp2 transcription

Our data argues that the function of Akt at regulating Skp2 levels is primarily through the regulation of Skp2 protein stability. Substitution of the Ser72 residue to a non-phosphorylatable Ala created a much more unstable Skp2 protein. Conversely, the phospho-mimetic S72D/S75D Skp2 mutant is much more stable than the wild-type protein. This is likely due to the ability of Akt to affect Cdh1-mediated Skp2 degradation since the S72D/S75D mutant resists degradation, and because Akt failed to protect the S72A mutant from degradation. Moreover, in cells where Akt1 is depleted, reduced phosphorylation of Ser72 in Skp2 correlates with a marked decrease in Skp2 levels. This process is likely due to enhanced Skp2 degradation by Cdh1, since inactivation of Cdh1 resulted in restoration of Skp2 to a level comparable to control siRNA-treated samples. Our results do not necessarily disagree with a recent report which showed that activation of PI 3-K and Akt also influences Skp2 mRNA levels [45]. It has been shown that Skp2 is a downstream target of E2F-1 [46], and thus the regulation of its expression at the level of transcription is higher in cells in which Rb is defective. It has also been reported that activation of Akt promotes the binding of E2F-1 to the proximal Skp2 promoter [47]. Therefore, Skp2 upregulation in most human cancers might be due to a synergistic action of upregulated Skp2 mRNA levels with a concomitant evasion of Cdh1-mediated degradation.

Does Akt regulate other APC/Cdh1 substrates other than Skp2?

For most SCF/F-box complexes, the regulation of substrate recognition occurs at the level of the substrate, such that the F-box protein will usually not recognize its downstream substrates without a specific combination of phosphorylation events. On the other hand, the interaction of Cdh1 and Cdc20 with their substrates usually does not require any post-translational modifications [48]. In this case, the regulation of APC activity occurs primarily on the APC complex itself. Phosphorylation of Cdc20 by Plk and Cdc2/Cyclin B is required for the activation of the APC/Cdc20 complex while phosphorylation of Cdh1 by the Cdk2/Cyclin A complex terminates Cdh1 activity by dissociating Cdh1 from the APC core subunits [49]. Recently, it was shown that APC/Cdh1 ubiquitinates and degrades its substrates with different kinetics, and the preference of degradation order depends on the relative processivity of substrate multiubiquitination by APC/Cdh1 [50].

Our finding provides another unique mechanism for the selective degradation of Cdh1 downstream targets. In all the known Cdh1 substrates tested so far, only Skp2 expression levels are affected by Akt signaling. This provides a novel link between p27 degradation and Akt activation, both of which can be induced by growth factors. Unlike most E3 ubiquitin ligases, Cdh1 is active in a low-kinase state during early G1 and in quiescent cells. Thus it is assumed that Cdh1 plays a major role in the maintenance of quiescence [51]. It is also known that most cells in human tissues are in a differentiated (post-mitotic) state, and in many cases loss of this differentiated state plays a major role in promoting tumorigenesis, and p27 has been demonstrated to be a critical player. KPC has been shown to play an important role in triggering the degradation of p27 in the early G1 phase [52]. Furthermore, Skp2-governed p27 degradation is critical for the timely activation of Cdk2 activity, and subsequent transition through the restriction point. Consistent with a previous report [5], our own studies show that activation of

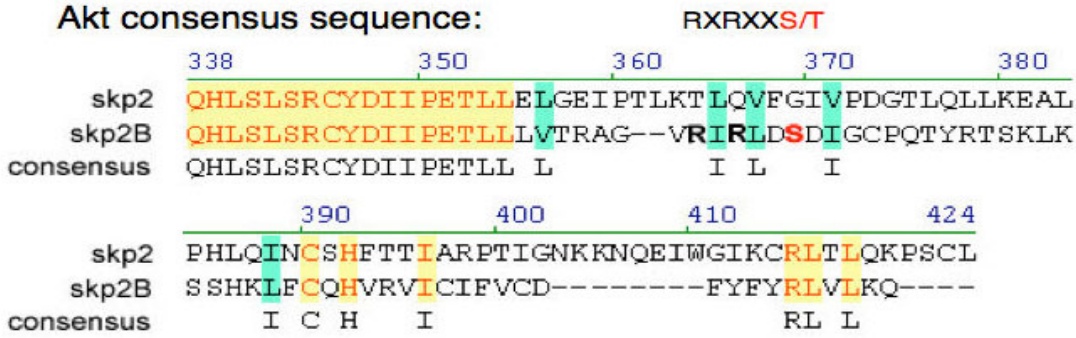

Figure 2
Sequence alignment of the Skp2 and Skp2B proteins reveals that there is an additional potential Akt phosphorylation site at the C-terminus of the Skp2B protein.

Akt upon serum stimulation allows for the early induction of Skp2, which correlates with p27 disappearance. This protective mechanism mediated by the Akt pathway is very similar to the Cdk2/cyclin E complex, which protects Cdc6 from Cdh1-mediated destruction [53]. Thus, our finding expands our knowledge of how specific kinase cascades could influence Cdh1-governed proteolysis. In contrast to the SCF complex, where phosphorylation triggers substrate recognition, we propose that a specific phosphorylation event by Akt, in addition to CKI, disables the degradation mediated by APC/Cdh1. Upon Cdk2/cyclin E phosphorylation, the interaction between Cdh1 and Cdc6 is reduced [53]. Similar to their finding, we found that the interaction between Skp2 and Cdh1 is also affected by Akt phosphorylation.

The next outstanding question is whether Akt could affect the stability of other APC/Cdh1 substrates. We found that manipulation of Akt activity does not affect the expression of other known Cdh1 substrates we examined, including Cdc20, Plk-1, Geminin, Cdc6, Securin, cyclin A and cyclin B [3,49]. These results indicate that Akt might only specifically affects the destruction of a subgroup of Cdh1 substrates including Skp2 and others that are still unidentified. We used the Scansite program to screen all known Cdh1 downstream targets, and found that except for Cdc25A and DNMT1, none of the other Cdh1 substrates contains the canonical Akt site. Therefore, it is interesting to further investigate whether depletion of Akt1 results in decreased expression of both Cdc25A and DNMT1. Furthermore, similar to what Akt does to Skp2, whether elevated Akt activity protects Cdc25A and DNMT1 from Cdh1-mediated destruction.

Akt promotes Skp2 cytoplasmic translocation

The Akt pathway functions to promote both cell survival and cell growth by inactivating many of its downstream substrates [23]. Interestingly, by the same token, phosphorylation of Akt substrates usually results in their cytoplasmic translocation. In the case of p27, p21 and FOXO proteins, the Akt phosphorylation site is proximal to the Nuclear Localization Sequence (NLS) and when phosphorylated creates a binding site that can be recognized by the 14-3-3 proteins. Recruitment of 14-3-3 results in the masking of the NLS and subsequent cytoplasmic translocation [54]. We also observed an interaction of 14-3-3 with Skp2 in cells expressing activated Akt. However, Ser72 phosphorylation per se is not sufficient for recruitment of 14-3-3. Thus it is possible that 14-3-3 is recruited to Skp2 after additional Akt phosphorylation events, or indirectly through interaction with Akt. The NLS of Skp2 resembles that of SV40 T antigen, which is recognized by the importin complex, the only difference being that Skp2 harbors the Ser72 Akt site. We also demonstrated that deletion of the NLS results in cytoplasmic localization, indicating the requirement of this sequence for nuclear import. Moreover, *in vitro* biochemical analyses demonstrated that deletion of the NLS disrupts the association between Skp2 and importin, providing further evidence that the specific association between Skp2 and the importin complex requires the NLS. Alternatively, the phosphorylation of serine or threonine residues by specific kinases within the NLS could impair the interaction between the importin complex and the NLS [55,56]. In keeping with this notion, we also demonstrated that phosphorylation of human Skp2 by Akt at Ser72 greatly reduces the interaction between Skp2 and importin. It is possible that both of these mechanisms contribute to the cytoplasmic translocation of Skp2 subsequent to Akt phosphorylation. This process is likely to be important for Skp2 function because Akt phosphorylation of the Skp2 downstream targets p27, p21 and FOXO1 also promotes their cytoplasmic localization, thus terminating their function. Our findings add a new dimension to this model by suggesting that Akt-

induced cytoplasmic translocation of Skp2 may lead to elevated degradation of its downstream targets by the cytoplasmic SCF/Skp2 complex. Lin et al suggested that cytoplasmic Skp2 plays an important role in promoting cellular motility [42]. It is well documented that cytoplasmic Skp2 is frequently observed in more advanced breast and prostate cancer. Therefore, it is plausible that cytoplasmic Skp2 activity promotes metastasis. It is important to further elucidate the novel substrates for cytoplasmic Skp2, which will provide important insight for developing new anti-cancer treatments.

Akt isoform specificity in the regulation of Skp2 protein stability

Interestingly, our data points to Akt isoform specificity in the regulation of Skp2 protein stability. Using siRNA's, we found that Akt1, but not Akt2, is responsible for phosphorylation of Skp2 at Ser72, and in turn, modulation of its protein stability. Furthermore, we demonstrated that when overexpressed in 293T cells, human Skp2 specifically interacts with endogenous Akt1, but not Akt2. Although the precise mechanism by which Akt1 can, whereas Akt2 cannot, signal to Skp2 has yet to be defined, and likely mechanisms include the localization of distinct Akt isoforms in cells and tissues. It is plausible that the nuclear localization of Akt1 which has been observed in some cell lines may allow it to interact with nuclear Skp2 and promote nuclear export, and that the more cytoplasmic localization of Akt2 may restrict its accessibility to Skp2. Although these and other possibilities have yet to be tested, our data is consistent with the recent finding that only Akt1 promotes G1 progression, DNA synthesis and proliferation of C2 myoblasts, whereas Akt2 is primarily required for exit from mitosis [38].

Is Skp2B a better substrate for Akt?

Skp2 cytoplasmic localization has been observed in many clinical tumor samples and is correlated with aggressive malignancy and poor diagnosis [15,57-60]. Our results offer a molecular mechanism for the cytoplasmic localization of Skp2. Furthermore, since elevated Akt also inactivates the Bad, Caspase-9 and FOXO proteins to allow tumor cells to evade the apoptosis pathway, cancer cells with cytoplasmic Skp2 localization tend to be more advanced. Recently, another novel Skp2 splicing isoform (Skp2B) was identified, which possesses many distinct molecular properties because it differs from the Skp2 protein at the carboxyl-terminus [61]. One major difference is that in contrast with the nuclear localization of Skp2, Skp2B localizes to the cytoplasm [58]. Comparing the Skp2 and Skp2B sequences showed that Skp2B contains the identified NLS, and does not have an obvious nucleus export signal (NES) sequence at its unique carboxyl-terminus. However, Skp2B contains an additional high probability Akt site in this region (Figure 2). This might make Skp2B an extremely effective strong substrate for Akt, which could result in cytoplasmic translocation by unknown mechanism.

Concluding remarks

Collectively, we and others provide evidence that Akt directly controls Skp2 stability and oncogenic activity. We identified one major Akt phosphorylation site on Skp2 at Ser72 that is located within a putative Nuclear Localization Sequence (NLS). We demonstrated that phosphorylation of Ser72 by Akt1, but not Akt2, promotes Skp2 cytoplasmic translocation, likely due to a disruption of the NLS. This finding provides an explanation for previous observations whereby cytoplasmic Skp2 staining is detected in tissues from advanced breast and prostate cancer. Thus in cells released from serum-starvation, elevated Akt activity in the early G1 phase protects Skp2 from constitutive degradation by Cdh1. Hence, our finding expands our knowledge of how specific kinase cascades influence proteolysis governed by the APC/Cdh1 complex. In addition, these findings provide insight into how the activated PI3-K/Akt pathway leads to elevated Skp2 expression and subsequent enhanced p27 destruction in human cancers, providing further evidence that elevated Akt activity and cytoplasmic Skp2 expression may be causative for breast and prostate cancer progression. These results may provide a rationale to develop specific Akt1 inhibitors as efficient anti-cancer drugs.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WW drafted the manuscript. DG designed the figures. All authors read and approved the final manuscript.

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Rictor Forms a Complex with Cullin-1 to Promote SGK1 Ubiquitination and Destruction

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SUMMARY

The Rictor/mTOR complex (also known as mTORC2) plays a critical role in cellular homeostasis by phosphorylating AGC kinases such as Akt and SGK at their hydrophobic motifs to activate downstream signaling. However, the regulation of mTORC2 and whether it has additional function(s) remain largely unknown. Here, we report that Rictor associates with Cullin-1 to form a functional E3 ubiquitin ligase. Rictor, but not Raptor or mTOR alone, promotes SGK1 ubiquitination. Loss of Rictor/Cullin-1-mediated ubiquitination leads to increased SGK1 protein levels as detected in Rictor null cells. Moreover, as part of a feedback mechanism, phosphorylation of Rictor at T1135 by multiple AGC kinases disrupts the interaction between Rictor and Cullin-1 to impair SGK1 ubiquitination. These findings indicate that the Rictor/Cullin-1 E3 ligase activity is regulated by a specific signal relay cascade and that misregulation of this mechanism may contribute to the frequent overexpression of SGK1 in various human cancers.

INTRODUCTION

The mammalian target of Rapamycin (mTOR) plays a critical role in regulation of cellular homeostasis, cell growth, and survival pathways by acting as a sensor for upstream inputs from multiple growth-promoting signals that are then transduced to downstream effectors (Guertin and Sabatini, 2007; Reiling and Sabatini, 2006). In order to fulfill this complex regulatory function, the mTOR kinase assembles into at least two distinct complexes termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Guertin and Sabatini, 2007; Reiling and Sabatini, 2006). These two multicomponent subcomplexes differ both structurally and functionally and signal to distinct downstream substrates. mTORC1 is composed of mTOR, Raptor, PRAS40 (proline-rich Akt substrate of 40 kilodaltons), and mLST8/GβL (G protein β subunit-like protein). The best-characterized mTORC1 kinase substrates include S6K (p70 S6 ribosomal kinase) and 4E-BP1 (phosphorylated 4E-binding protein). The mTORC2 complex includes mTOR, Rictor, mLST8/GβL, PROTOR (protein observed with Rictor-1)/PRR5, and Sin1 (Jacinto et al., 2006; Shiota et al., 2006). mTORC2 phosphorylates the hydrophobic motif of Akt at Ser473 (Sarbassov et al., 2005) and SGK1 at Ser422 (García-Martínez and Alessi, 2008), leading to full kinase activation. Because aberrant activation of Akt is a hallmark of many types of cancers (Manning and Cantley, 2007), hyperactivation of mTORC2 activity has been implicated in cancer progression (Guertin and Sabatini, 2007).

The activity of the mTORC1 complex is highly regulated in cells exposed to growth factors and nutrients. In response to mitogens, activation of PI 3-K (phosphoinositide 3-kinase) leads to phosphorylation of the TSC2 (tuberous sclerosis 2) and PRAS40 proteins by Akt, culminating in activation of mTORC1 (Manning and Cantley, 2007). The activity of mTORC1 can also be stimulated by the Rag GTPase in response to nutrient stimulation (Sancak et al., 2008). In addition, phosphorylation of Raptor by AMPK (5′ AMP-activated protein kinase) in response to a low-energy state provides a negative regulatory mechanism to repress mTORC1 activity (Gwinn et al., 2008). Although mTORC2 is a key upstream kinase complex that functions to control Akt phosphorylation and downstream signaling, relatively little is known regarding the regulation of mTORC2. Recent studies indicate that the mTOR complexes might be multifunctional and contain activities other than protein kinases. For example, Raptor has been shown to form a complex with the Cullin-4 E3 ligase, and this complex might be critical for mTOR kinase activity (Ghosh et al., 2008). Rictor has also been shown to associate with Cullin-4, although, unlike Raptor, it is not a WD40 repeat-containing protein (Ghosh et al., 2008). However, additional function(s) for Rictor and mTORC2 remain largely unknown.

The serum and glucocorticoid-inducible kinase (SGK) belongs to the AGC (protein kinase A, G, and C) family of kinases, and its activity is stimulated by growth factors (Lang et al., 2006). There are three closely related family members designated SGK1, SGK2, and SGK3 (Loffing et al., 2006). One of the

best-characterized SGK1 downstream targets is Foxo3a, which is involved in the regulation of apoptosis (Brunet et al., 2001). SGK1 has also been indicated in the regulation of Na^+ retention through phosphorylation of Nedd4-2 to impair its ability to degrade the epithelial Na^+ channel (ENaC) (Debonneville et al., 2001; Ichimura et al., 2005). SGK isoforms share ~80% similarity in the kinase domains with other AGC family kinases, including Akt and S6K. In vitro SGK recognizes the same phosphorylation consensus motif (RXRXXS/T, in which X represents any amino acid) as Akt and S6K (McCormick et al., 2004). However, unlike Akt and S6K, whose expression is relatively stable, SGK1 is a short-lived protein whose stability is controlled by the ubiquitin-proteasome pathway (Loffing et al., 2006). Both the Nedd4-2 (Brickley et al., 2002; Zhou and Snyder, 2005) and CHIP (C-terminal Hsc70-interacting protein) E3 ligases have been shown to ubiquitinate SGK1 (Belova et al., 2006). The first 60 amino acids of SGK1 are critical for Nedd4-2-mediated destruction of SGK1 (Bogusz et al., 2006; Brickley et al., 2002). In addition to growth factor stimulation, cellular stresses, including osmotic stress, heat shock, oxidative stress, and ultraviolet irradiation, induce SGK1 expression by transcriptional mechanisms and thus influence cell survival, proliferation, and differentiation (Lang and Cohen, 2001; Loffing et al., 2006). However, it remains unclear how SGK1 destruction by Nedd4-2 and CHIP is regulated by these cellular stresses and whether other unidentified E3 ligase(s) play a critical role in governing SGK1 destruction in response to these signaling events. Finally, both Akt and SGK are frequently amplified and/or overexpressed in cancers (Sahoo et al., 2005), although the underlying molecular mechanisms are unknown.

Here, we evaluate the mechanism by which Rictor controls SGK1 stability. We found that, by specific association with Cullin-1 and Rbx1, Rictor forms a functional E3 ubiquitin ligase complex that promotes the ubiquitination of SGK1, but not Akt1 or S6K1. We also show that the AGC kinases phosphorylate Rictor at T1135 to disrupt the Rictor/Cullin-1 complex and impair its E3 ligase activity and subsequent SGK1 ubiquitination. Our findings demonstrate a kinase-independent function for the Rictor protein and provide a mechanistic explanation for the observed elevation of SGK1 expression in various human tumors.

RESULTS

SGK1 Protein Expression Is Regulated by Rictor

SGK1 is an unstable protein, and previous studies have shown that, in response to serum and growth factors, activation of PI 3-K leads to the induction of SGK1 (Park et al., 1999). Induced SGK1 expression occurs partially through the increased transcriptional levels of SGK1 mRNA and partially through other layers of posttranslational regulation (Loffing et al., 2006). However, the exact molecular mechanism(s) remain elusive. Recent studies have shown that the Rictor-containing mTORC2 complex phosphorylates SGK1 at S422 in the hydrophobic motif to fully activate SGK1 kinase activity (García-Martínez and Alessi, 2008). We therefore first investigated whether Rictor signaling can also influence SGK1 expression. We found that *rictor*$^{-/-}$ MEFs have elevated SGK1 expression levels under serum-deprived conditions (Figure 1A). In agreement with previous studies (Webster et al., 1993), re-addition of serum led to a significant induction of SGK1 protein in both wild-type and *rictor*$^{-/-}$ MEFs. Elevated SGK1 expression in *rictor*$^{-/-}$ MEFs has been reported (Huang et al., 2009), although the underlying molecular mechanism has not been characterized. Loss of Rictor is the primary mechanism for the observed elevation of SGK1 expression because reintroduction of wild-type Rictor into *rictor*$^{-/-}$ MEFs dramatically reduced SGK1 expression (Figure 1B). Consistent with this, depletion of Rictor, but not Raptor, in HeLa cells also led to an accumulation of SGK1 protein, primarily in the early G1 phase of the cell cycle (Figure 1C). However, Akt1 and S6K1 expression was not affected by depletion of endogenous Rictor in HeLa cells (Figure 1C). Depletion of Rictor does not significantly affect SGK1 mRNA levels (Figures S1A–S1C available online), indicating that posttranslational modification(s) might contribute to the regulation of SGK1 by Rictor. In support of this notion, depletion of Rictor led to a significant increase in SGK1 half-life (Figure S1H). Furthermore, depletion of other mTORC2 complex components mTOR or Sin1 resulted in a decrease, rather than accumulation, of SGK1, indicating that Rictor might regulate SGK1 abundance independent of its mTORC2 kinase activity (Figure S1D). Moreover, inactivation of the P-I3K pathway by both LY and Wortmannin treatment leads to a significant decrease in SGK1 expression, and this process was blocked by MG132 treatment (Figures S1E–S1F), supporting an involvement of the 26S-proteasome pathway. More importantly, LY-induced SGK1 degradation is partially blocked after depletion of the endogenous Rictor (Figure 1D), further supporting a potential physiological role for Rictor in SGK1 stability control.

To better understand how Rictor controls SGK1 expression, we examined the difference between wild-type and *rictor*$^{-/-}$ MEFs in their endogenous signaling pathways responding to growth factor addition (insulin in Figure S1I and IGF in Figure S1J). We found that *rictor*$^{-/-}$ MEFs are defective in activating Akt, as evidenced by the lack of induction of the pSer473-Akt signal (Guertin et al., 2006; Shiota et al., 2006). Of interest, SGK1 expression in wild-type MEFs peaks 0.5–1 hr post-growth factor addition and then fades away at later time points (2–4 hr). In contrast, there is elevated SGK1 expression in *rictor*$^{-/-}$ cells and no significant decrease of SGK1 at the late time points. These data suggest a possible role for Rictor in promoting the destruction of SGK1 at late time points following growth factor addition (Lang and Cohen, 2001; Webster et al., 1993).

Rictor Promotes SGK1 Ubiquitination in a Cullin-1-Dependent Manner

In support of the finding that Rictor regulates SGK1 stability, we detected an interaction between Rictor and SGK1 (Figure 2A). Ubiquitin immunoblotting reveals that Rictor can promote SGK1 ubiquitination (Figure S2A). To exclude the possible contribution of other known SGK1 E3 ligases, including Nedd4-2 and CHIP, we used a deletion mutant of SGK1 that lacks the amino-terminal 60 amino acids (Δ60-SGK1), which cannot be efficiently ubiquitinated by Nedd4-2 and CHIP (Brickley et al., 2002; Zhou and Snyder, 2005). We used a cell-based ubiquitination assay to address whether Rictor and other mTOR components could promote SGK1 ubiquitination. Surprisingly,

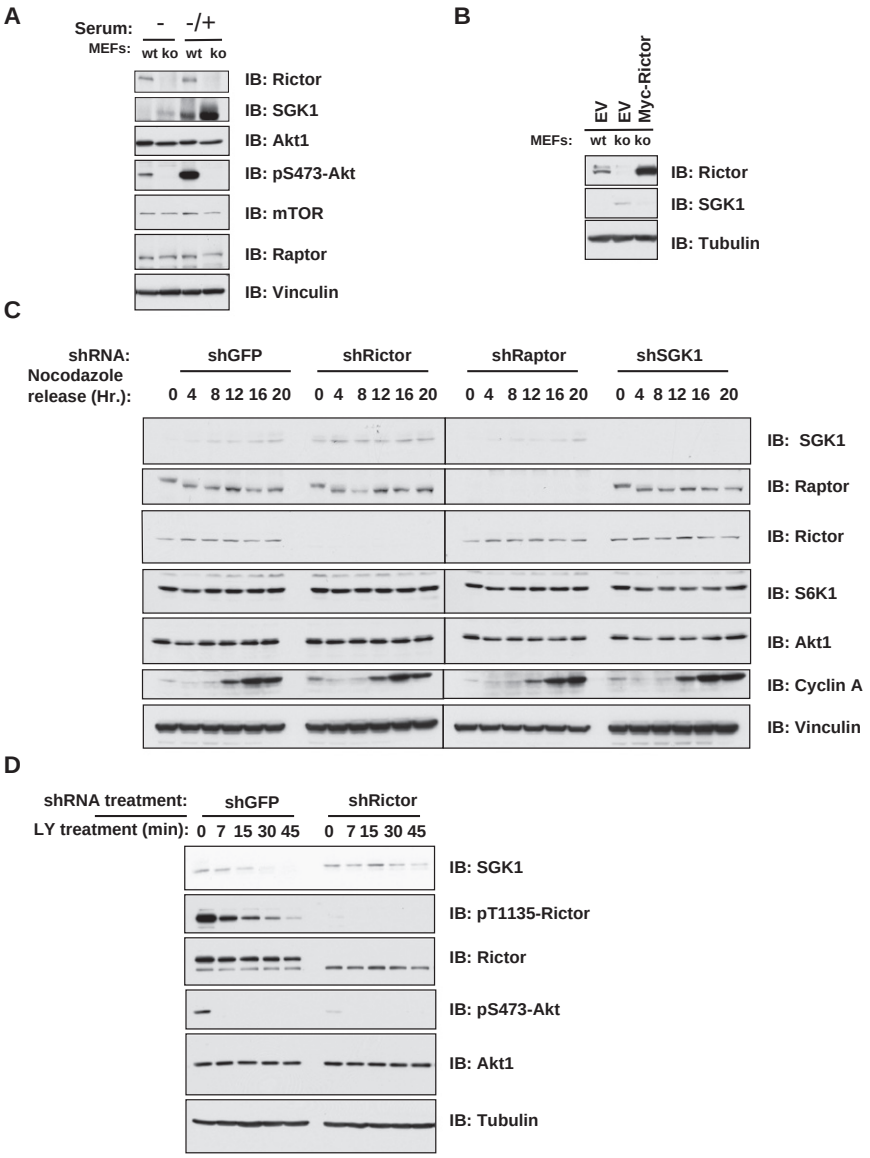

Figure 1. SGK1 Expression Is Regulated by the Rictor Pathway

(A) Whole-cell lysates were isolated from wild-type or *rictor*$^{-/-}$ mouse embryonic fibroblasts (MEFs) in serum starvation conditions for 24 hr. In another experimental condition, 10% FBS was added to the serum-starved cells for 1.5 hr before harvesting. Equal amounts of whole-cell lysates were immunoblotted with the indicated antibodies.

(B) Immunoblot analysis of wild-type or *rictor*$^{-/-}$ MEFs transfected with the Myc-Rictor plasmid (with empty vector as a negative control) together with pBabe-Puro retroviral empty vector constructs. Twenty-four hours posttransfection, the cells were treated with 1 μg/ml puromycin for 48–72 hr to kill the nontransfected cells prior to collecting the whole-cell lysates for immunoblots.

(C) HeLa cells were infected with the indicated lentiviral shRNA constructs and selected with 1 μg/ml puromycin to eliminate the noninfected cells. The resulting HeLa cell lines were arrested in the M phase by incubation with nocodazole for 18 hr and then released into the G1 phase. At the indicated time points, cell lysates were collected for immunoblot analysis.

(D) HeLa cells were infected with lentiviral shRictor construct (with shGFP as a negative control) and selected with 1 μg/ml puromycin to eliminate the noninfected cells. The resulting cell lines were treated with 20 μM LY294002, and at the indicated time points, whole-cell lysates (WCL) were collected for immunoblot analysis.

See also Figure S1.

E3 ligase activity toward SGK1 is unique to Rictor, as neither Raptor nor mTOR promotes SGK1 ubiquitination (Figure 2B). SGK1 was recently identified to be a specific downstream substrate of mTORC2 (Rictor), but not mTORC1 (Raptor) (García-Martínez and Alessi, 2008). Therefore, we reasoned that SGK1 may specifically interact with Rictor, but not Raptor, which could explain why Raptor does not promote SGK1 ubiquitination, despite the fact that Raptor was recently shown to exist in a complex with Cullin 4 (Ghosh et al., 2008). However, as shown in Figure S2B, Raptor does not promote the ubiquitination of either S6K1 or Akt1, consistent with the widely accepted notion that, unlike SGK, both Akt and S6K are relatively stable proteins. Furthermore, short-term or long-time treatment with Rapamycin does not affect the ability of Rictor to promote SGK1 ubiquitination, even though long-term Rapamycin treatment led to a significant decrease of SGK1 Ser422 phosphorylation by mTORC2, arguing that Rictor-mediated SGK1 ubiquitination might be mTORC kinase activity independent (Figure 2C).

Because Rictor itself does not contain the ring finger, PHD, or HECT domain that possesses intrinsic E3 ligase activity, it is possible that Rictor associates with other cofactor(s) to form an E3 ligase complex. The Cullin-Ring complex comprises the largest family of E3 ubiquitin ligases (Petroski and Deshaies, 2005). Thus, we first determined which Cullin family member might contribute to SGK1 ubiquitination. We found that overexpression of a dominant-negative Cullin-1 allele (but not DN Cullin-2, -3, or -4) specifically impairs the ability of Rictor to promote SGK1 ubiquitination (Figure 2D). In keeping with this finding, we showed that Rictor specifically interacts with Cullin-1, but not other Cullin family members Cullin-2, -3 and -4 (Figures 2E, S2C, and S2D). These data are indicative of a role for Cullin-1 in SGK1 ubiquitination.

Rictor Forms a Complex with Cullin-1 and Rbx1 to Promote SGK1 Destruction

In support of a possible physiological role for both Rictor and Cullin-1 in regulating SGK1 stability, we detected the endogenous interaction between Rictor and Cullin-1 (Figures 3A and

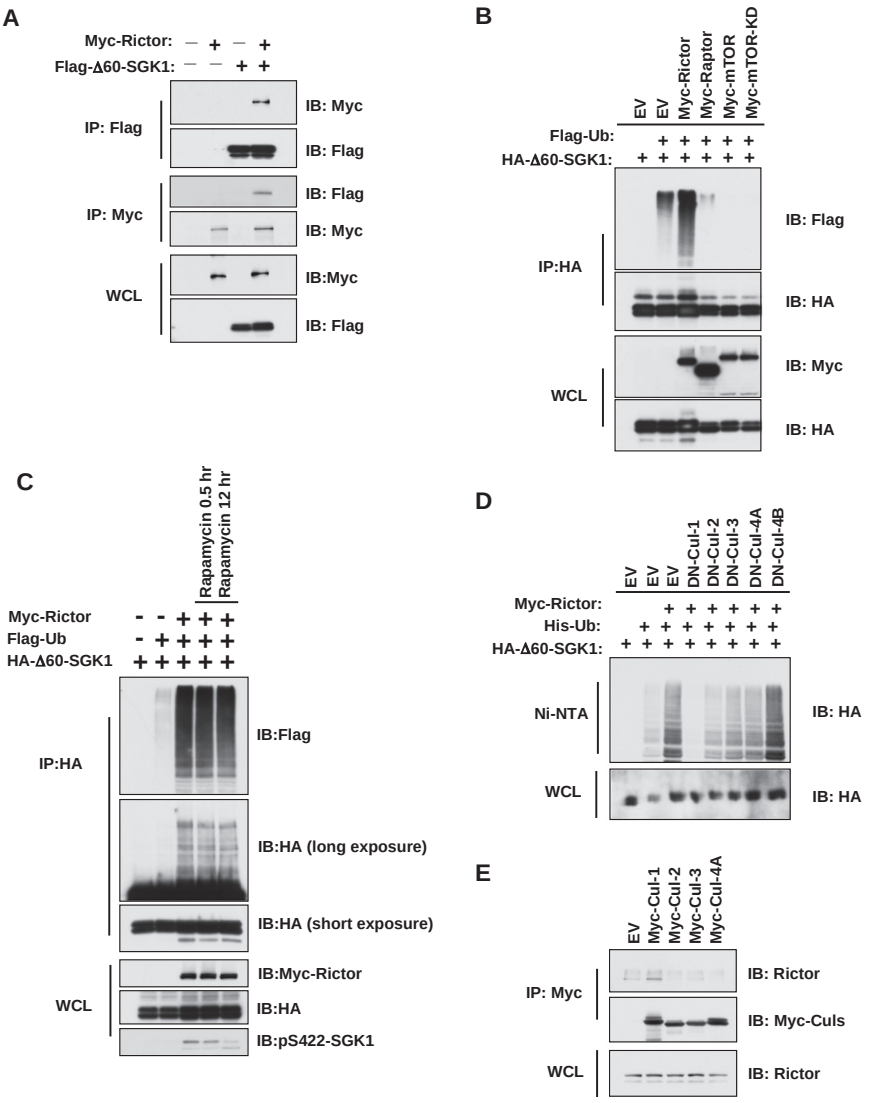

Figure 2. Rictor Promotes SGK1 Ubiquitination in a Cullin-1-Dependent Manner

(A) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with Flag-Δ60-SGK1 and Myc-Rictor constructs. Thirty hours posttransfection, cells were treated with 10 μM MG132 for 10 hr to block the proteasome pathway before harvesting.

(B) Immunoblot analysis of whole-cell lysates (WCL) and anti-HA immunoprecipitates derived from 293T cells transfected with the indicated plasmids. Twenty hours posttransfection, cells were treated with the proteasome inhibitor MG132 overnight before harvesting.

(C) Immunoblot (IB) analysis of whole-cell lysates (WCL) and anti-HA immunoprecipitates of 293T cells transfected with the indicated plasmids. Twenty hours posttransfection, cells were treated with the proteasome inhibitor MG132 overnight or 100 nM Rapamycin for the indicated period of time before harvesting.

(D) Expression of a dominant-negative form of Cullin-1 blocks the ability of Rictor to promote SGK1 ubiquitination. Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with HA-Δ60-SGK1, Myc-Rictor, and His-Ub in the presence of various dominant-negative forms of Cullin family members. Twenty hours posttransfection, cells were treated with the proteasome inhibitor MG132 overnight before harvesting. The whole-cell lysates were collected in EDTA-free lysis buffer, and the His pull-down was carried out in the presence of 8 M Urea to disrupt possible protein interactions.

(E) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with the indicated Myc-Cullin constructs. Thirty hours posttransfection, cells were treated with 10 μM MG132 for 10 hr to block the proteasome pathway before harvesting. See also Figure S2.

3B). Furthermore, the endogenous interaction between Rictor and Cullin-1 could be detected in both CHAPS buffer that preserves the mTORC2 complex (Figures 3A and S3A) and NP-40-containing EBC buffer (Figure 3B), a condition that has been shown to disrupt the mTORC2 complex (Hara et al., 2002; Kim et al., 2002), indicating that the intact Rictor/mTOR complex might not be required for interaction with Cullin-1. In support of this idea, we showed that Rictor, but not Sin1 or mTOR, specifically interacts with endogenous Cullin-1 (Figure 3C). Furthermore, Cullin-1 only specifically interacts with Rictor, but not Raptor, or other known mTORC2 components, including mTOR, GβL, and Sin1 (Figure 3D). Of note, Cullin-1 binds endogenous Rictor with similar intensity as Sin1 in both CHAPS and EBC buffer conditions (Figure S3D). Although mTOR interacts with Rictor more strongly than Cullin-1 in CHAPS buffer, mTOR/Rictor interaction is not detected in EBC buffer (Figure S3C) (Hara et al., 2002; Kim et al., 2002). These results suggest that Cullin-1 interacts with Rictor in vivo.

Cullin-1 is an extensively studied member of the Cullin family (Cardozo and Pagano, 2004; Harper et al., 2002; Nakayama and Nakayama, 2005). It complexes with Rbx1, Skp1, and various F box proteins to form a multiprotein SCF (Skp1, Cullin-1, F box protein) E3 ligase complex (Cardozo and Pagano, 2004; Schulman et al., 2000). To further understand the physiological components of the Rictor/Cullin-1 complex, we performed gel filtration chromatography. As shown in Figure S3G, we found that there might be two different pools of Rictor complex. One complex was estimated at a size greater than 600 KD (fractions 22–26), comigrating with activated mTOR (as evidenced by S2481 phosphorylation) and Sin1, which might represent the mTORC2 complex. The other Rictor-containing complex was detected at 300–400 KD (fractions 30–34) and comigrates with both Cullin-1 and Rbx1, but not Sin1. Because Sin1 is required for mTORC2 complex formation (Jacinto et al., 2006; Yang et al., 2006), the lack of Sin1 precludes a possible existence of the mTORC2 complex in these fractions. In contrast,

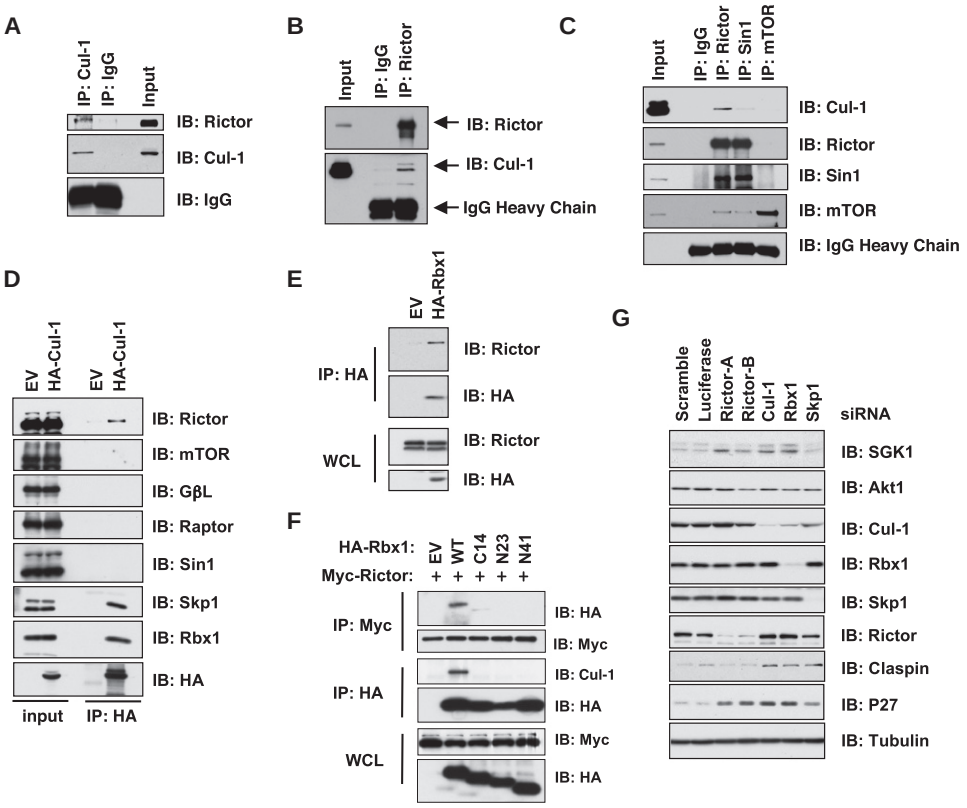

Figure 3. Rictor Interacts with Cullin-1 and Rbx1 In Vivo

(A) Immunoblot (IB) analysis of 293T cell whole-cell lysates (WCL) and anti-Cullin-1 immunoprecipitates (IP). Mouse IgG was used as a negative control for the immunoprecipitation procedure. WCL were collected with CHAPS buffer, and IPs were washed with CHAPS buffer.

(B) Immunoblot (IB) analysis of 293T cell whole-cell lysates (WCL) and anti-Rictor immunoprecipitates (IP). Rabbit IgG was used as a negative control for the immunoprecipitation procedure. WCL were collected with EBC buffer, and IPs were washed with NETN buffer.

(C) Immunoblot (IB) analysis of 293T cell whole-cell lysates (WCL) and anti-Rictor, anti-Sin1, and anti-mTOR immunoprecipitates (IP). Rabbit IgG was used as a negative control for the immunoprecipitation procedure. WCL were collected with EBC buffer, and IPs were washed with NETN buffer.

(D) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from HeLa cells transfected with the HA-Cullin-1 construct.

(E) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with HA-Rbx1 construct. Thirty hours posttransfection, cells were pretreated with 10 μM MG132 for 10 hr to block the proteasome pathway before harvesting.

(F) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with Myc-Rictor together with the indicated HA-Rbx1 constructs. Thirty hours posttransfection, cells were pretreated with 10 μM MG132 for 10 hr to block the proteasome pathway before harvesting.

(G) Immunoblot analysis of HeLa cells transfected with the indicated siRNA oligonucleotides. The control lanes are scrambled E2F-1 siRNA and siRNA against firefly luciferase. siRNA, short interfering RNA.

See also Figure S3.

one Raptor complex (mTORC1 complex) is detected, estimated to be greater than 600 KD and comigrating with mTOR. Another detected Raptor peaks at around 180–250 KD (fractions 36–40), which might represent the free Raptor monomer. Consistent with the coimmunoprecipitation data (Figure 3D), there is no detected comigration between Raptor and Cullin-1 at fractions 29–34 with anticipated size (around 250–400 KD), corresponding to a possible Raptor/Cullin-1 complex.

Consistent with the gel filtration experiment, we observed an interaction between Rictor and Rbx1 (Figures 3E and S3H), which recruits the E2 enzyme to the E3 ubiquitin ligase complex. Using a series of Rbx1 mutants that are unable to interact with Cullin-1, we showed that the presence of Cullin-1 is required for Rbx1 and Rictor interaction (Figures 3F and S3I). However, we found that, under ectopic overexpression conditions, Rictor does not interact with Skp1 (Figures S3J–S3K). In line with these biochemical evidences, depletion of Rictor, Cullin-1, and Rbx1, but not Skp1, leads to increased SGK1 expression (Figures 3G, S3F, and S3L). Furthermore, depletion of endogenous Skp1 does not significantly affect the interaction between Rictor and Cullin-1 (Figure S3M) nor the ability of Rictor to promote SGK1 ubiquitination in vivo (Figure S3N). Altogether, these data support the hypothesis that a unique complex composed of Rictor, Cullin-1, and Rbx1 (and possibly other unknown partners) is involved in regulating SGK1 abundance. However, it requires further investigation to fully understand the role of Skp1 in this process.

We next evaluated the relationship between the mTORC2 kinase complex and the Rictor/Cullin-1 ubiquitin E3 ligase complex. In agreement with a recent study (Huang et al., 2009),

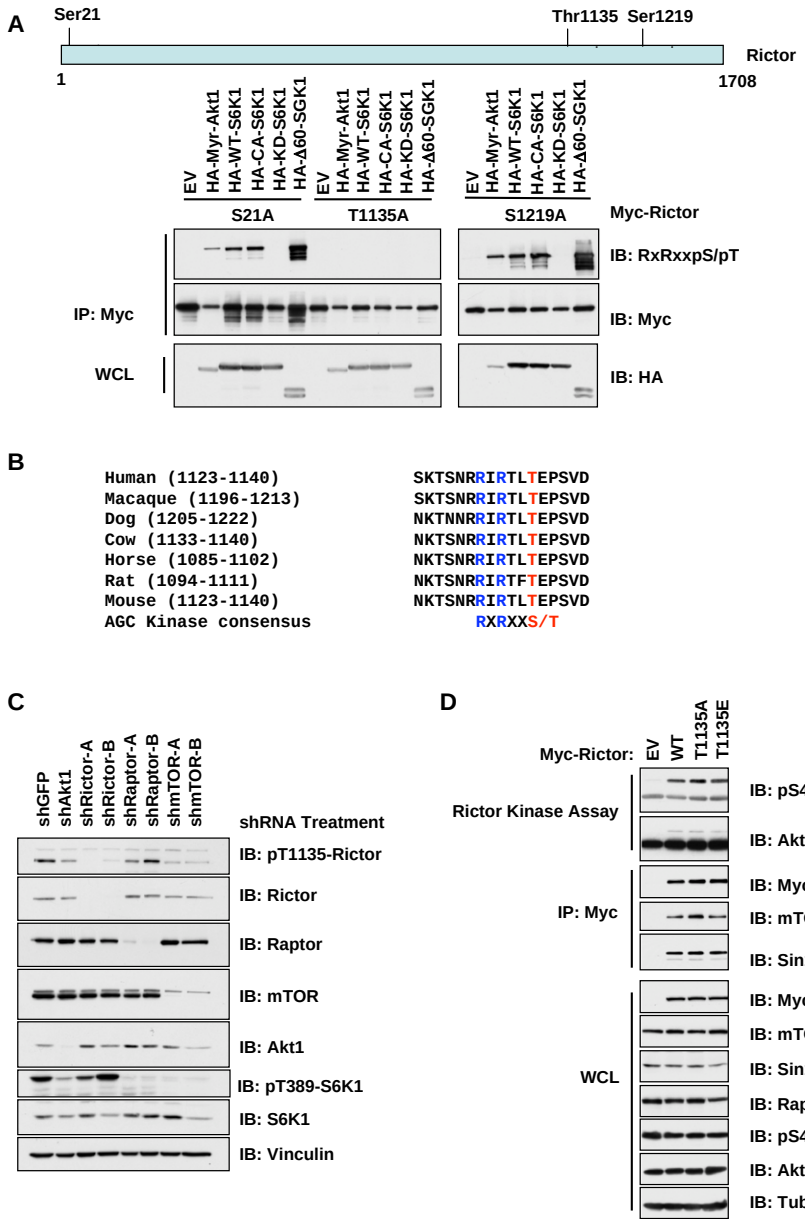

Figure 4. Rictor Is Phosphorylated In Vivo at T1135

(A) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with the indicated Myc-Rictor constructs together with various HA-tagged AGC family of kinases.

(B) Sequence alignment of the putative Rictor T1135 phosphorylation site across different species.

(C) HeLa cells were infected with the indicated lentiviral shRNA constructs and selected with 1 μg/ml puromycin to eliminate the noninfected cells. Cell lysates were collected for immunoblot analysis.

(D) 293T cells were transfected with the indicated Myc-tagged Rictor constructs. Thirty-six hours posttransfection, whole-cell lysates were collected, and the mTORC2 complex was purified by Myc immunoprecipitation. The Myc immunoprecipitates were incubated in vitro with the biochemically purified inactive Akt1 (from Upstate) in the presence of ATP and the kinase reaction buffer. Thirty minutes later, the reaction was stopped by the addition of the loading buffer. Akt1 phosphorylation status was examined by immunoblot analysis.

See also Figure S4.

both the amino and carboxyl termini of Rictor are required for association with mTORC2 (Figures S3O–S3P). In contrast, although the carboxyl terminus of Rictor containing the T1135 site (see Figures 4A and 4B) is sufficient for interaction with Cullin-1, deletion of this region did not lead to a complete loss of interaction with Cullin-1, indicating that the N terminus of Rictor also plays a role in mediating Cullin-1 interaction (Figure S3Q). However, only the full-length Rictor, but not amino- or carboxyl-terminal truncation mutants, forms a functional E3 ligase/Cullin-1 complex to promote SGK1 ubiquitination (Figure S3R). These data support a possible model whereby association of Rictor with distinct subsets of cofactors can assemble into distinct complexes to modulate a functional output, although the precise molecular mechanism that switches Rictor from a kinase mode to an ubiquitin ligase mode remains to be determined.

Rictor Is Phosphorylated by AGC Kinases at T1135

Although Rictor is a key regulator of both Akt and SGK1, how its activity is regulated remains largely unknown. In agreement with recent reports (Dibble et al., 2009; Julien et al., 2009; Treins et al., 2009), we found that S6K1 phosphorylates Rictor at T1135 (Figures 4A, 4B, and S4A). Furthermore, ectopically expressed Rictor is also phosphorylated in cells by Akt1 and SGK1 (Figure 4A-B). Phosphorylation of the Rictor T1135 site in vivo was further confirmed by mass spectrometry analysis (Figure S4B).

To further understand the regulatory mechanism of Rictor T1135 phosphorylation, we developed a phospho-specific antibody against this motif (Figures S4C and S4D). Depletion of mTOR, but not Raptor in HeLa cells, leads to a significant reduction of T1135 Rictor phosphorylation, indicating that both mTORC1 and mTORC2 activities are possibly involved (Figure 4C). In keeping with this finding, inhibition of PI 3-K with LY or Wortmannin treatment is more efficient than inhibition of Akt with a specific Akt inhibitor or inhibition of mTORC1 with Rapamycin in reducing T1135 phosphorylation (Figures S1E–S1G and S4E). Moreover, although depletion of S6K1 leads to a reduction in T1135 phosphorylation, depletion of Akt1 or SGK1 delivers similar effects, and depletion of any individual AGC kinase does not significantly affect endogenous Rictor T1135

phosphorylation as depletion of mTOR does (data not shown). This possibly indicates that all three AGC family kinases might function in a redundant manner toward Rictor phosphorylation at T1135. A similar mechanism has been reported for Foxo3a phosphorylation by AGC kinases (Tran et al., 2003; Vogt et al., 2005).

Phosphorylation of Rictor at T1135 Disrupts the Cullin-1/Rictor Interaction

Next we investigated the consequence of Rictor T1135 phosphorylation on mTORC2 complex activity. Although Rictor T1135 phosphorylation leads to recruitment of 14-3-3 (Figures S5A–S5C), it does not disrupt the mTORC2 complex nor does it significantly affect the ability of Rictor/mTOR to phosphorylate Akt1 in vitro (Figure 4D). These results indicate that, unlike Raptor (Gwinn et al., 2008), phosphorylation of Rictor at T1135 by AGC kinase might not significantly affect its kinase activity and likely affects distinct Rictor functions. Following this lead, we next investigated whether it affects the Rictor/Cullin-1 E3 ligase complex. As shown in Figure S5E, using gel filtration chromatography, we detected comigration of p-T1135-Rictor with an active form of mTOR (p-S2481) corresponding to the mTORC2 complex (fractions 14–20). However, p-T1135-Rictor did not comigrate with Cullin-1 at 300–400 KD (fractions 21–26). Similarly, wild-type-Rictor, but not phosphomimetic T1135E-Rictor, comigrated with Cullin-1 (Figures 5A and S5E), indicating that phosphorylation of Rictor at T1135 might serve to disrupt the Rictor/Cullin-1 complex. In support of this notion, Cullin-1 was found to specifically interact with nonphosphorylated Rictor species (Figures 5B and 5C), and mutation of Thr1135 to phosphomimetic Glu disrupts the interaction between Rictor and Cullin-1 (Figures 5D, 5E, and S5F).

To gain a better understanding of how phosphorylation of Rictor at T1135 functions physiologically in vivo, we used a stable HeLa cell line expressing HA-Cullin-1 at levels comparable to endogenous Cullin-1 (Figures S5I and S5J) and performed a series of time course experiments to investigate how physiological manipulation of Rictor T1135 phosphorylation affects Cullin-1/Rictor interaction. As shown in Figure 5F, insulin addition into serum-starved HeLa cells leads to activation of the S6K and Akt kinase pathways (as shown by the increased p-S473-Akt and p-T389-S6K signals), which results in increased Rictor phosphorylation at T1135, coupled with a decrease in Cullin-1/Rictor interaction and enhanced 14-3-3/Rictor interaction and, consequently, increased SGK1 abundance.

These data indicate that Rictor phosphorylation at T1135 might function in a similar fashion as the phosphomimetic T1135E mutant in abolishing Rictor interaction with Cullin-1. However, T1135E failed to interact with 14-3-3 (Figures S5A and S5C), indicating that, unlike Raptor phosphorylation by AMPK (Gwinn et al., 2008), simple recruitment of 14-3-3 after Rictor phosphorylation is not the only cause to disrupt Rictor/Cullin-1 interaction. We noticed that disruption of the endogenous interaction between 14-3-3 and Rictor by the R18 peptides leads to a sharp decrease in phosphorylation of Rictor at T1135 (Figure S5D). This indicates that 14-3-3 interaction might serve as a mechanism to protect T1135 from dephosphorylation, which has been described for Bad (Chiang et al., 2003; Datta et al., 1997) and Foxo3a (Singh et al., 2010). In this sense, it is the phosphorylation event at Rictor T1135, rather than the interaction with 14-3-3, that results in disruption of the Cullin-1/Rictor complex. Although the T1135E Rictor mutant does not interact with 14-3-3, it behaves similarly as Rictor phosphorylated at T1135, both introducing a negative charge to cause a possible local conformation change to impair Rictor interaction with Cullin-1. In support of this, a peptide composed of 200 amino acids surrounding the T1135 site is sufficient to interact with Cullin-1 in vitro, whereas the T1135E mutation leads to a significant reduction in their interaction in vitro (Figure 5E).

In a reciprocal set of experiments, we treated HA-Cullin-1-expressing cells cultured in 10% FBS-containing DMEM medium with LY290042 to inactivate PI 3-K and then monitored how this affects the Cullin-1/Rictor interaction. LY treatment induced a time-dependent decrease of Rictor T1135 phosphorylation, presumably due to the inactivation of S6K and Akt, which correlates with a decrease in 14-3-3 interaction and an increase in Rictor interaction with Cullin-1, resulting in decreased SGK1 abundance (Figure 5G). Therefore, in both experimental conditions, Rictor phosphorylation at T1135 is inversely correlated with its ability to interact with Cullin-1.

Phosphorylation of Rictor at T1135 Reduces the Ability of Rictor to Ubiquitinate SGK1

As a result of reduced interaction with Cullin-1, T1135E Rictor is defective in promoting SGK1 ubiquitination (Figures 6A–6C and S6A). Because T1135E has similar affinity as wild-type Rictor in binding to SGK1 (Figures S5H and S6B), the impaired E3 ligase activity toward ubiquitination of SGK1 might primarily be due to the disruption of the Cullin-1/Rictor association. Moreover, in keeping with impaired E3 ligase activity, compared with wild-type Rictor, T1135E Rictor is also compromised in promoting SGK1 degradation (Figures 6D and S6C) and thus is more potent at promoting S phase entry (Figure 6E). Consistent with the above findings, loss of the PTEN tumor suppressor, which activates the PI 3-K/Akt pathway, also results in elevated SGK1 expression (Figures S6D and S6E). This is directly correlated with increased Rictor phosphorylation at T1135. This argues that loss of PTEN may lead to elevated Akt and S6K activities, resulting in enhanced Rictor phosphorylation at T1135 and, in turn, an impaired ability of the Rictor/Cullin-1 complex to degrade SGK1. In summary, these data demonstrate that, in addition to complexing with mTOR to form the mTORC2 complex as a means to phosphorylate Akt and SGK, by complexing with Cullin-1 and Rbx1, Rictor might have an additional function as an E3 ligase complex that controls the stability of SGK1 and likely additional targets (Figure 7). We also show that the E3 ligase activity of Rictor is subject to negative regulation by a variety of AGC kinases. Because PI 3-K positively regulates AGC kinase activity, frequent hyperactivation of this signaling axis might contribute to the elevated SGK1 expression levels detected in various cancers (Figure 7).

DISCUSSION

The data presented above provide experimental evidence for a possible function of Rictor in the ubiquitination of SGK1.

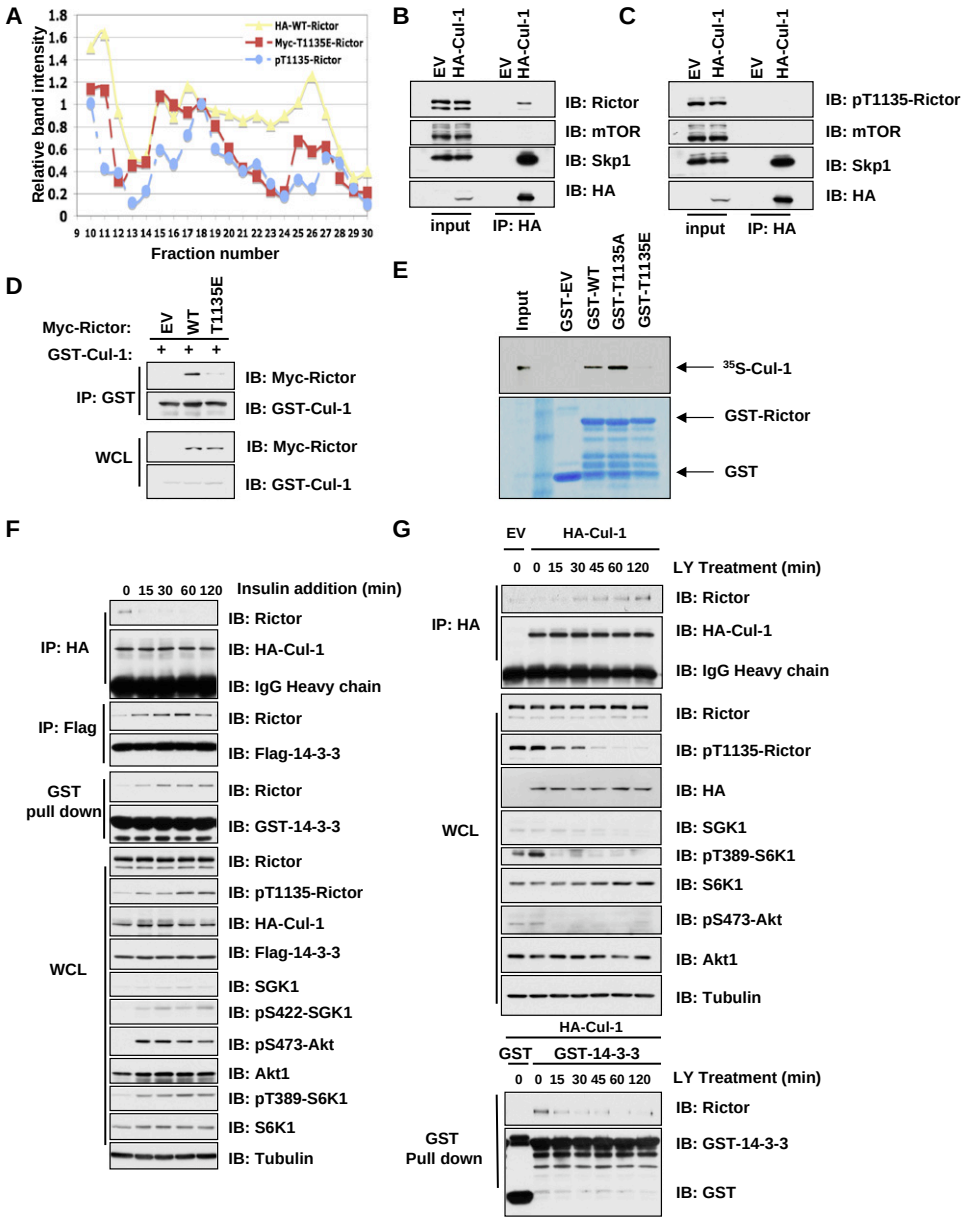

Figure 5. Phosphorylation of Rictor at T1135 Disrupts the Interaction between Rictor and Cullin-1

(A) HeLa cells were transiently transfected with HA-WT-Rictor and Myc-T1135E-Rictor constructs. Thirty hours posttransfection, whole-cell lysates were collected in CHAPS buffer and subjected to gel filtration chromatography. The relative band intensities for HA-WT-Rictor, Myc-T1135E-Rictor, and p-T1135-Rictor at the indicated fractionations were quantitated. The original immunoblots were shown in Figure S5E.

(B and C) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with the HA-Cullin-1 construct. Anti-Rictor (B) or anti-p-T1135-Rictor (C) immunoblot analysis was performed on two identical immunoprecipitations to illustrate that Cullin-1 does not interact with T1135-phosphorylated Rictor species.

(D) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with the indicated Myc-Rictor and GST-Cullin-1 constructs.

(E) Autoradiography of ^{35}S-labeled Cullin-1 bound to the indicated GST-fusion proteins.

(F) HA-Cullin-1 expressing HeLa cells transiently transfected with the Flag-14-3-3 construct. Eighteen hours posttransfection, cells were serum starved for 24 hr. After the addition of 100 nM insulin, whole-cell lysates (WCL) were collected at the indicated time points for immunoblot analysis with the indicated antibodies, for Flag-IP and GST pull-down assays to determine Rictor/14-3-3 interaction, and for HA-IP to determine Rictor/Cullin-1 interaction.

(G) HA-Cullin-1-expressing HeLa cells were treated with 20 μM LY294002. At the indicated time points, whole-cell lysates (WCL) were collected for immunoblot analysis with the indicated antibodies, for HA-IP to determine Rictor/Cullin-1 interaction, and for GST pull-down assays to determine Rictor/14-3-3 interaction. See also Figure S5.

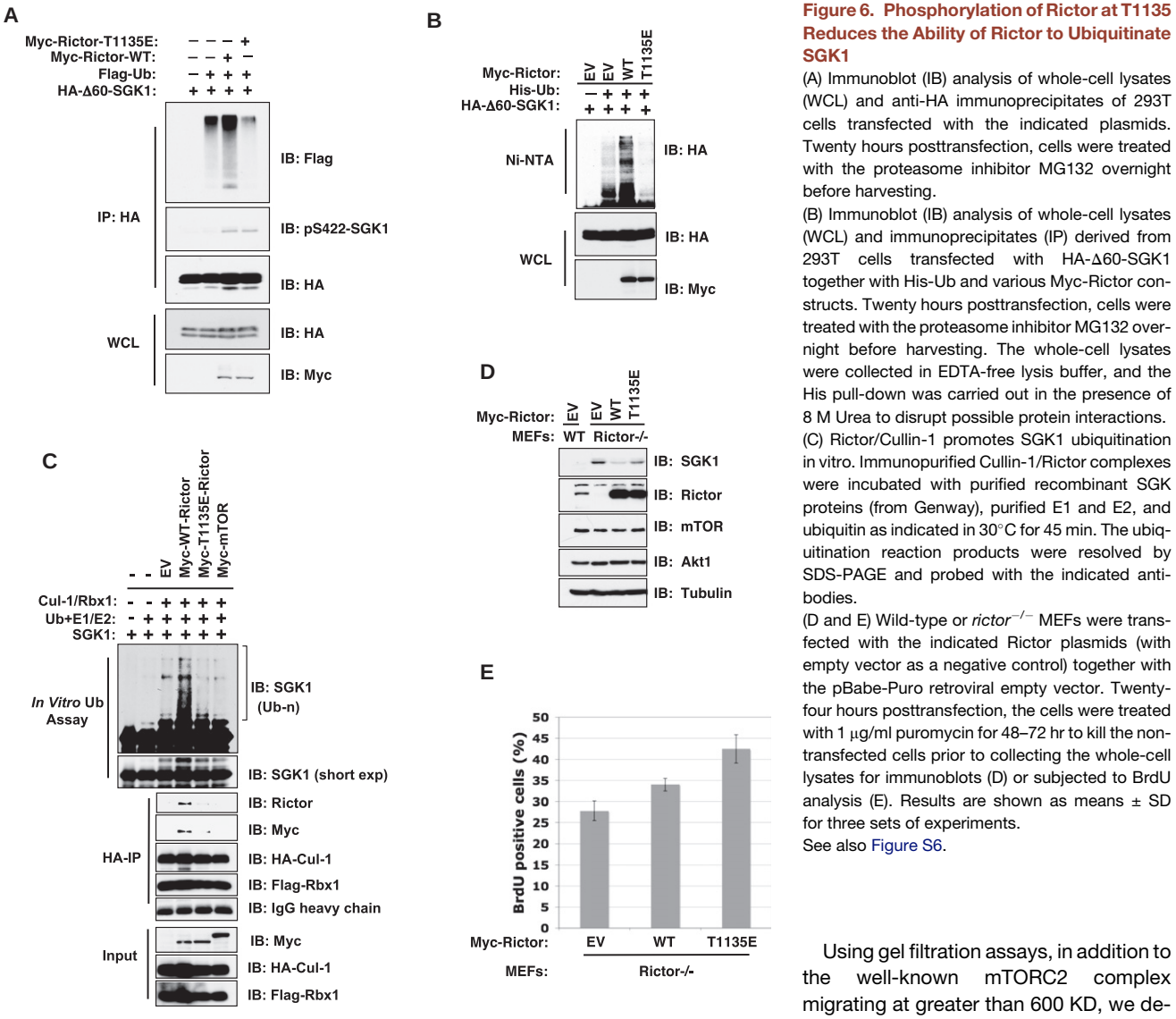

Figure 6. Phosphorylation of Rictor at T1135 Reduces the Ability of Rictor to Ubiquitinate SGK1

(A) Immunoblot (IB) analysis of whole-cell lysates (WCL) and anti-HA immunoprecipitates of 293T cells transfected with the indicated plasmids. Twenty hours posttransfection, cells were treated with the proteasome inhibitor MG132 overnight before harvesting.

(B) Immunoblot (IB) analysis of whole-cell lysates (WCL) and immunoprecipitates (IP) derived from 293T cells transfected with HA-Δ60-SGK1 together with His-Ub and various Myc-Rictor constructs. Twenty hours posttransfection, cells were treated with the proteasome inhibitor MG132 overnight before harvesting. The whole-cell lysates were collected in EDTA-free lysis buffer, and the His pull-down was carried out in the presence of 8 M Urea to disrupt possible protein interactions.

(C) Rictor/Cullin-1 promotes SGK1 ubiquitination in vitro. Immunopurified Cullin-1/Rictor complexes were incubated with purified recombinant SGK proteins (from Genway), purified E1 and E2, and ubiquitin as indicated in 30°C for 45 min. The ubiquitination reaction products were resolved by SDS-PAGE and probed with the indicated antibodies.

(D and E) Wild-type or *rictor*$^{-/-}$ MEFs were transfected with the indicated Rictor plasmids (with empty vector as a negative control) together with the pBabe-Puro retroviral empty vector. Twenty-four hours posttransfection, the cells were treated with 1 μg/ml puromycin for 48–72 hr to kill the nontransfected cells prior to collecting the whole-cell lysates for immunoblots (D) or subjected to BrdU analysis (E). Results are shown as means ± SD for three sets of experiments.

See also Figure S6.

To date, the only function attributed to Rictor in the mTORC2 complex is phosphorylation of the hydrophobic motifs of downstream targets such as Akt and SGK. Our data point to a specific association of Rictor with Cullin-1 and Rbx1 as part of a possible functional E3 ubiquitin ligase complex (Figure 7).

Although our results indicate that mTOR may not be required for Rictor E3 ligase activity (Figure 2B), it remains unclear whether these two complexes share common scaffolding proteins and what the crosstalk is between these two complexes. Recent studies demonstrate that many other kinase complexes also possess E3 ligase activity (Lu et al., 2002; Maddika and Chen, 2009). Because many ubiquitination processes require a prior phosphorylation event, the coupling of both the kinase and the E3 ligase in proximity provides for more efficient destruction (Carrano et al., 1999). It is possible that there are unknown Rictor ubiquitination substrate(s) that require prior phosphorylation by Rictor.

Using gel filtration assays, in addition to the well-known mTORC2 complex migrating at greater than 600 KD, we detected a second possible Rictor-containing complex, comigrating with Cullin-1 and Rbx1 at 300–400 KD (Figure S3G). This finding was supported by endogenous coimmunoprecipitation assays showing that only Rictor, but not other mTORC2 complex components, including mTOR, GβL, and Sin1, associates with Cullin-1 (Figures 3A–3D and S3A). We also found that, although Sin1 could efficiently immunoprecipitate endogenous Rictor, it could not immunoprecipitate endogenous Cullin-1 as Rictor IP does (Figure 3C). Because Sin1 is required for formation of the mTORC2 complex (Jacinto et al., 2006; Yang et al., 2006), these results suggest that Rictor might exist in two distinct complexes, the Sin1-containing mTORC2 complex and the Cullin-1-containing complex. This was further supported by the observation that depletion of Rictor, Cullin-1, and Rbx1, but not mTOR, Raptor, or Sin1, leads to upregulation of SGK1 (Figures S1D and 3G). However, additional studies are required to fully understand the possible role of mTOR and other TORC2 complex components in Rictor-mediated SGK1 turnover.

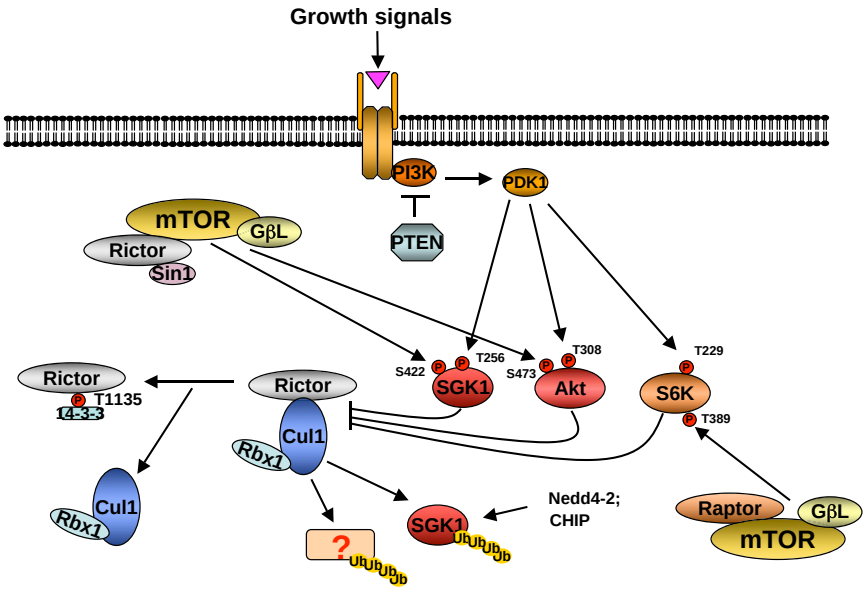

Figure 7. Proposed Model for the Rictor/Cullin-1 Pathway to Control SGK1 Turnover

Our data also demonstrate that, unlike Rictor, Raptor does not promote the ubiquitination of Akt1, SGK1, and S6K1 (Figures 2B and S2B). This suggests that the E3 ligase activity might be unique to the Rictor/Cullin-1 complex. However, under ectopic expression conditions, Skp1 is not detected in the Rictor/Cullin-1 complex (Figures S3J and S3K). It is known that Skp1 serves as a bridging molecule to hold Cullin-1 and the F box protein in a complex. The possible lack of Skp1 in the Cullin-1/Rictor complex suggests that Rictor might play a role equivalent to Skp1. However, further experimental investigation is required to fully understand the role of Skp1 for the Rictor/Cullin-1 complex to ubiquitinate SGK1, and more studies are also needed to determine whether a specific F box protein is involved in this process.

Although SGK1 has been shown to be degraded by other E3 ligases, including Nedd4-2 and CHIP, in both cases, it is not known how the destruction is regulated and whether it is mediated by PI 3-K signaling. Our data suggest an alternative mechanism whereby multiple AGC kinases can negatively regulate Rictor/Cullin-1 E3 ligase activity without affecting its kinase activity, and this suggests a positive feedback loop to boost SGK1 activity poststimulation. However, more studies are required to determine whether the ability of Nedd4-2 and CHIP to degrade SGK1 is also affected by the PI 3-K/Akt signaling. SGK1 overexpression has been reported in multiple cancers, including breast cancer. Of greatest interest, although SGK1 has been suggested to have redundant functions with Akt, simultaneous overexpression of both Akt and SGK1 has also been reported in breast cancers (Sahoo et al., 2005). Our work suggests that, because SGK1, but not Akt, is subject to ubiquitination by the Rictor/Cullin-1 complex (Figure S6A), elevated Akt activity might block the function of Rictor/Cullin-1, leading to accumulation of SGK1 (data not shown and Figure S6F). Collectively, our results provide insight into how Rictor can influence SGK1 signaling by promoting its ubiquitination. Furthermore, we define a feedback mechanism that can negatively regulate the E3 ligase activity of the Rictor/Cullin-1 complex. This provides functional insight into Rictor regulation, as well as mechanistic information regarding the SGK1 stability controlled by the PI 3-K pathway, and how misregulation of this process contributes to SGK1 overexpression in human cancers.

EXPERIMENTAL PROCEDURES

Cell Culture and Cell Synchronization

Cell culture, including synchronization and transfection, has been described (Gao et al., 2009). Lentiviral shRNA virus packaging and subsequent infection of various cell lines were performed according to the protocol described previously (Boehm et al., 2005). *rictor*$^{-/-}$ MEFs and control MEFs were kind gifts from Dr. Mark Magnuson (Shiota et al., 2006). Kinase inhibitors LY294002 (Sigma, L9908), Rapamycin (Calbiochem, 553210), Wortmannin (Sigma, 95455), and Akt 1/2 Inhibitor VIII (Calbiochem, 124018) were used as indicated.

In Vitro Kinase Assay

mTORC2 in vitro kinase assay was performed as described previously (Sarbassov et al., 2005).

In Vivo Ubiquitination Analysis

Cells were transfected with a plasmid encoding HA-Δ60-SGK1 along with Flag- or His-tagged ubiquitin. myc-Rictor or other expression vectors were co-transfected to assess their effects on SGK ubiquitination. Thirty-six hours after transfection, 10 μM MG132 was added to block proteasome degradation, and cells were harvested in EBC buffer or denaturing buffer (6 M Guanidine-HCL, 0.1 M NaH_2PO_4, 10 mM Tris-HCL, and 10 mM immidazole [pH 8.0]) containing protease inhibitor. Whole-cell lysates (2 mg) were incubated with Flag beads or Ni-NTA resin for 4–10 hr, followed by washing four times with NETN buffer or denaturing washing buffer (8 M urea, 0.1 M NaH_2PO_4, 10 mM Tris-HCL, and 10 mM immidazole [pH 6.3]). Then the washed pellet was boiled in SDS-containing lysis buffer and resolved on SDS-PAGE.

In Vitro Ubiquitination Assay

The in vitro ubiquitination assays were performed as described previously (Jin et al., 2005). To purify the Cullin-1/Rictor E3 ligase complex, 293T cells were transfected with vectors encoding HA-Cullin-1, Myc-Rictor (WT or T1135E), and Flag-Rbx1. The Cullin-1/Rictor (E3) complexes were purified from the whole-cell lysates using HA-agarose beads. Purified, recombinant SGK protein (purchased from Genway) was incubated with purified Cullin-1/Rictor (E3) complexes in the presence of purified, recombinant active E1, E2 (UbcH5a and UbcH3), ATP, and ubiquitin. The reactions were stopped by the addition of 2 × SDS-PAGE sample buffer, and the reaction products were resolved by SDS-PAGE gel and probed with the indicated antibodies.

Mass Spectrometry Analysis to Detect Rictor T1135 Phosphorylation In Vivo

293T cells were transiently transfected with Myc-Rictor plasmid, and 18 hr posttransfection, cells were grown in serum-deprived conditions for 24 hr. Thirty minutes after addition of insulin, whole-cell lysates were collected to perform Myc immunoprecipitation. Myc immunoprecipitates were resolved

on SDS-PAGE and visualized by colloidal Coomassie blue. The band containing Rictor was excised and treated with DTT to reduce disulfide bonds and iodoacetamide to derivatize cysteine residues. In-gel digest of the protein was performed with trypsin. The peptides were extracted from the gel, and phosphopeptides were enriched by immobilized metal affinity chromatography (IMAC) and then analyzed by nanoscale-microcapillary reversed phase liquid chromatography tandem mass spectrometry (LC-MS/MS), essentially as described previously (Villén and Gygi, 2008). Peptides were separated across a 37 min gradient ranging from 4% to 27% (v/v) acetonitrile in 0.1% (v/v) formic acid in a microcapillary (125 μm × 18 cm) column packed with C_{18} reverse-phase material (Magic C18AQ, 5 μm particles, 200 Å pore size, Michrom Bioresources) and online analyzed on the LTQ Orbitrap XL hybrid FTMS (Thermo Scientific, Bremen, Germany). For each cycle, one full MS scan acquired on the Orbitrap at high-mass resolution was followed by ten MS/MS spectra on the linear ion trap XL from the ten most abundant ions. MS/MS spectra were searched using the SEQUEST algorithm (Eng et al., 1994) against a database created on the basis of a protein sequence database containing the sequence of Rictor, of common contaminants, such as human keratin proteins with static modification of cysteine carboxymethylation, dynamic modification of methionine oxidation and serine, threonine, and tyrosine phosphorylation. All peptide matches were filtered based on mass deviation, tryptic state, XCorr, and dCn and were confirmed by manual validation. The reliability of site localization of phosphorylation events was evaluated using the Ascore algorithm (Beausoleil et al., 2006).

Gel Filtration Chromatography for Separation of Rictor Complexes

Two 10 cm plates of HeLa cells were washed with phosphate-buffered saline, lysed in 0.5 ml of CHAPS lysis buffer (25 mM HEPES [pH 7.4], 150 mM NaCl, 1 mM EDTA, and 0.3% CHAPS), and filtered through a 0.45 μm syringe filter. Total protein concentration was 6 mg/ml. 500 μl of lysate was loaded onto a Superdex 200 10/300 GL column (GE Lifesciences Cat. No. 17-5175-01) (Figure S3G). The experiment in Figure S5E was performed with this same column attached in series to a Superose 6 10/300 GL (GE Lifesciences Cat. No. 17-5172-01). The sample was separated in the Superose 6 column first and then the Superdex 200 column. The gel filtration beads in each column have different size exclusion characteristics that complement each other and allow separation of very large and also smaller proteins. Chromatography was performed using an AKTAFPLC (GE Lifesciences Cat. No. 18-1900-26), and protein complexes were resolved by eluting with the same CHAPS buffer at 0.5 ml/min for 3 hr. Eluent was collected in either 250 μl (Figure S3G) or 500 μl (Figure S5E) fractions. 50 μl aliquots of these fractions were loaded onto SDS-PAGE minigels for SDS-PAGE and western blot analysis of the fractionated protein complexes. Prior to running cell lysates, the molecular weight resolution of the columns was first estimated by running native molecular weight markers (urease ~545 kDa, mouse monoclonal IgG ~180 kDa, human serum albumin ~68 kDa) and determining their retention times on Coomassie-stained SDS-PAGE protein gels.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.molcel.2010.08.016.

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